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TITLE OF THESIS ..The Uptake and Degradation of Bacterial Ribo-

..nucleic Acids by Ehrlich Ascites Cells.....

DEGREE FOR WHICH THESIS WAS PRESENTED ..Master of Science.....

YEAR THIS DEGREE GRANTED ..1974.....

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# THE UPTAKE AND DEGRADATION OF BACTERIAL RIBONUCLEIC ACIDS BY EHRLICH ASCITES CELLS

BY

JOHN WAI-HONG LAM

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

FALL , 1974

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "The Uptake and Degradation of Bacterial Ribonucleic Acids by Ehrlich Ascites Cells" submitted by John Wai-hong Lam in partially fulfilment of the requirements for the degree of Master of Science.

# DEDICATION

To my Mother and Father

# ABSTRACT

The uptake of high molecular weight RNA and the degradation of the ingested RNA were studied with Ehrlich ascites tumour cells. Two cell types were used; cells with normal RNase activities, and cells with high acid and alkaline RNase II activities and undetectable levels of endogenous RNase inhibitor.

The high molecular weight RNA was prepared by incubating Pseudomonas aeruginosa in the presence of $^{14}C$ uracil. The RNA was extracted and purified by Bio-Gel P-60 chromatography. It was characterized and found to be 85 to 92% ribosomal RNA. To estimate the RNA uptake, Ehrlich ascites cells were incubated in "modified" Fischer's medium with 60 µg/ml labelled RNA at 37°. The radioactivity associated with the cells was determined after repeated washing of the cells by centrifugation and resuspension. The rate of RNA incorporation was linear for 2 hours and 7 - 9% of the total RNA was associated with the cells at this time. Most of this RNA was ingested by the cells; however, a small amount seemed to be rapidly adsorbed to the cell surface. This adsorption was enhanced when the cells were pretreated with DEAE-dextran. Increasing the RNA concentration in the medium to greater than 60 µg/ml did not result in an increase in the rate of RNA uptake. Low molecular weight RNA or RNA degradation products were found to be taken up at a lower rate than high molecular weight RNA at the same concentration.

Ehrlich ascites cells with high RNase activities incorporated less radioactive RNA than cells with normal RNase activities. This was probably due to the more rapid degradation of the RNA in the medium when cells with high RNase activities were used.

The stability of the incorporated RNA was determined by incubating the cells in the presence or absence of actinomycin D. The cells were then washed and precipitated with cold 4% perchloric acid. Actinomycin D had very little effect on the uptake of the labelled RNA. However, 75% of the radioactivity associated with the cells was acid-soluble when RNA synthesis was inhibited, whereas few acid-soluble degradation products were detected in the absence of actinomycin D. These observations indicated that the incorporated RNA was being rapidly degraded and that the degradation products were retained within the cells and could be used for synthesis of mammalian RNA. Polyacrylamide gel electrophoresis of RNA extracted from cells incubated continuously with radioactive RNA confirmed that the bacterial ribosomal RNA was rapidly degraded and utilized for mammalian RNA synthesis. The radioactivity appeared first in the mammalian 45S ribosomal presursor and then in the 28S and 18S ribosomal RNA. The increase in the radioactive RNA extracted from the cells with time was small compared to the increase in the total radioactivity associated with the cells. This also suggested that much of the bacterial RNA was degraded to small molecules which were not retained in the RNA extraction procedure.

Degradation of high molecular weight RNA incorporated by the cells could not be distinguished readily from incorporation of RNA which had been degraded in the medium. Therefore, in order to minimize the effect of extracellular RNA degradation, cells were incubated with bacterial RNA for 20 minutes, washed once at $0^{\circ}$ - $4^{\circ}$, and then reincubated without RNA for 60 minutes at $37^{\circ}$. The fate of the relatively intact RNA that was incorporated was then followed by polyacrylamide gel electrophoresis. The result of these experiments suggests that the Ehrlich ascites cells with normal RNase activities and the cells with

high RNase activities degraded RNA intracellularly at approximately the same rate. The RNA in the medium, however, was degraded at a faster rate when cells with high RNase activities were used.

ACKNOWLEGEMENTS

I am indebted to Dr. R. G. von Tigerstrom for what I have learned from him. I am also grateful to others in the Department of Microbiology who have given me help directly or indirectly in this project.

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| | |
|---|---|
| RNA | - ribonucleic acid |
| rRNA | - ribosomal ribonucleic acid |
| tRNA | - transfer ribonucleic acid |
| mRNA | - messenger ribonucleic acid |
| pre-rRNA | - precursor ribosomal ribonucleic acid |
| Hn- RNA | - heterogeneous nuclear ribonucleic acid |
| DNA | - deoxyribonucleic acid |
| poly A | - polyadenylic acid |
| poly I | - polyinosic acid |
| poly C | - polycytidylic acid |
| UMP | - uridine monophosphate |
| PCA | - perchloric acid |
| TCA | - trichloroacetic acid |
| Hepes | - N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid |
| SDS | - sodium dodecyl sulfate |
| DEAE- | - diethylaminoethyl- |
| EDTA | - ethylenediaminetetracetic acid |
| Tris | - tris(hydroxymethyl) aminomethane |
| DTT | - dithiothreitol |
| TEMED | - N,N,N',N'-tetramethylethylenediamine |
| mCi | - millicurie |
| μCi | - microcurie |
| M | - molar |
| mM | - millimolar |

| | |
|---|---|
| nm | - nanometer |
| ml | - milliliter |
| µl | - microliter |
| µg | - microgram |
| mA | - milliampere |
| i.d. | - internal diameter |
| UV | - ultraviolet |
| A | - absorbance |
| OD | - optical density |
| S | - Svedberg unit of the sedimentation coefficient |
| ng | - nanogram |
| ppm | - parts per million |

# INTRODUCTION

This study was carried out in order to investigate the functions of acid and alkaline RNase II and the endogenous inhibitor of alkaline RNase II in Ehrlich ascites cells. Two observations prompted these experiments. One was that Ehrlich ascites cells can be obtained with either normal RNase activities, or with high RNase activities resulting from *in vitro* treatment of the cells before transplantation with a cytostatic agent such as actinomycin D (Erbe *et al*, 1966; von Tigerstrom, 1972). The other observation was that high molecular weight RNA was taken up by Ehrlich ascites cells and that the ingested RNA was relatively stable (Niu *et al*, 1961; Niu, 1963; Juliano and Mayhew, 1972). It was hoped that a comparison could be made between the fate of the RNA ingested by cells with normal and with high RNase activities and that such a comparison would yield information about the physiological functions of acid RNase II, alkaline RNase II and the endogenous inhibitor of alkaline RNase II.

## Characteristics of Mammalian RNA Degrading Enzymes

A number of mammalian RNA degrading enzymes have been isolated and characterized. Their properties have been extensively studied *in vitro* but their physiological functions are poorly understood. With the current understanding of RNA metabolism, possible functions for some of the enzymes have been proposed. A summary of the characteristics of the main RNA degrading enzymes is shown in Table 1. The nomenclature of the enzymes is that used by Razzell (1967).

TABLE 1

A SUMMARY OF PROPERTIES OF RNA DEGRADING ENZYMES IN MAMMALIAN CELLS

| Enzyme | Subcellular Distribution | Substrates | Products | Heat Stability | Acid Stability | Metal Ion Requirement | pH Optimum |
|---|---|---|---|---|---|---|---|
| Exo-RNase I | Nuclei | RNA<br>Poly A | Nucleoside-5'-P | Low | Low | $Mg^{++}$ | 7.2-7.6 |
| Endo-RNase I | Nuclei | RNA<br>Poly A | Nucleoside-5'-P<br>Oligonucleotide-5'-P | Low | Low | $Mg^{++}$ | 7.2-7.6 |
| Acid RNase II | Lysosomes<br>Microsomes | RNA | Nucleoside-3'-P<br>Oligonucleotide-3'-P | Low | High | None | 4.8-5.8 |
| Alkaline RNase II | Cytoplasmic<br>Nuclei | RNA | Nucleotide-3'-P<br>Oligonucleotide-3'-P | High | High | None | 7.0-8.3 |
| Phospho-diesterase I | Microsomes | RNA<br>DNA<br>Oligonucleotides | Nucleoside-5'-P | Low | Low | $Mg^{++}$ | 9.2 |
| Phospho-diesterase II | Microsomes<br>Soluble | RNA<br>DNA<br>Oligonucleotides | Nucleoside-3'-P | Low | Low | None | 6.0 |

RNase I activities have been reported to be associated with nuclei of many mammalian cells and this activity in the nuclei of mouse liver cells and Ehrlich ascites cells is due to an endo-RNase I and an exo-RNase I (Lazarus and Sporn, 1967). Both enzymes have a pH optimum of 7.2 to 7.6, have a requirement for $Mg^{2+}$ and are labile to acid and heat. Ribonucleic acids with a high degree of helical structure are resistant to attack by both enzymes. Exo-RNase I degrades single-stranded RNA in a processive manner to 5' mononucleotides from the 3' OH end (Sporn et al, 1969) and the emzyme is three times more active with poly A as the substrate than with RNA (Lazarus and Sporn, 1967). The endo-RNase I hydrolyzes single stranded RNA primarily to oligonucleotides with 5' phosphate ends (Heppel, 1966). An enzyme which also releases 5'phosphate terminated oligonucleotides with no marked base specificity has been isolated from the endoplasmic reticulum of rat liver and Ehrlich ascites cells (Morais and de Lamirande, 1965). However, it is insensitive to $Mg^{2+}$ and it is therefore likely to be different from the endo-RNase described by Heppel (1966).

Alkaline RNase II activity has been located in the mitochondrial, microsomal and supernatant fractions of liver cells (de Lamirande, 1959; Roth, 1957); in microsomes, zymogen granules and supernatant of pancreas (Dickman and Murrill, 1959); in mitochondrial and supernatant fractions of rat adrenals (Girija and Sreenivasan, 1966) and in the nuclei of Ehrlich ascites cells (Chakravorty and Busch, 1967). Difficulty in reaching an agreement about the exact location of the enzyme might, in part, be due to the adsorption of the enzyme to various cell components during the isolation procedure. Internucleotide bonds adjacent to the 3'phosphoryl groups of pyrimidine bases are hydrolyzed by the enzyme to

cyclic 2', 3' phosphates and then to 3' phosphates. Polynucleotides with purine bases are relatively resistant to attack and extensive hydrolysis of RNA by this enzyme leaves a resistant core of purine oligonucleotide terminated by a pyrimidine nucleoside-3' phosphate.

An endoribonuclease resembling alkaline RNase II has been isolated and puridied from nucleolar ribonucleoprotein particles by Prestayko et al (1973). The optimum conditions for the enzyme are neutral pH and 60°. It is resistant to heat and appears to have a specificity for pyrimidine bases. Its presence in the nucleolar fraction of Novikoff hepatoma cells suggests that it might be involved in the processing of 45S pre-ribosomal RNA.

An endogenous inhibitor of alkaline RNase II was first described by Roth (1956) and it has been detected in various tissues including Ehrlich ascites cells (Colter et al,1961; Chakravorty and Busch, 1967: von Tigerstrom, 1972). This inhibitor has been found to be a glycoprotein (Sirakov and Kochakian, 1969) whose pH optimum coincides with the pH optimum of the ribonuclease (7.0 - 8.3). The inhibitor can be inactivated by heat and acid whereas alkaline RNase II is highly resistant to both. It is also inactivated by heavy metal ions ($Hg^{2+}$, $Pb^{2+}$), sulfhydryl reactants such as p-chloromercuribenzoate and RNA(Roth, 1962). Sulfhydryl groups may therefore be essential for the ribonuclease inhibitor activity.

It has been suggested that alkaline RNase inhibitor levels may be one of the factors controlling cell proliferation and RNA turnover. In experiments done with fasted rats, Sheppard et al(1970) found that the increase in ribonuclease activity in liver extracts of fasted rats was due to changes in the concentration of an inhibitor and not to changes in the amount of the enzyme. The low ribonuclease activity in

livers of fed mice may result, possibly, from the presence of the glycoprotein inhibitor. It has been suggested that alkaline RNase II is responsible for the faster rate of degradation of rRNA in fasted rats (Hirsch and Hiatt, 1966). Kraft and Shortman (1970) concluded that high inhibitor/RNase ratios were associated with states of RNA accumulation and low ratios were associated with states of RNA catabolism. For example, phagocytes which have high catabolic activity were found to have very low levels of inhibitor and very high levels of ribonuclease activity. Furthermore, when human blood lymphocytes were stimulated into active dividing cells by phytohaemagglutinin, the inhibitor increased from undetectable to very high levels (Kraft and Shortman, 1970). However, in Novikoff hepatoma which proliferates extremely rapidly, RNase inhibitor activity is considerably reduced compared to normal tissues (Roth, 1962). This greatly reduced inhibitor activity was also observed in a slow growing hepatoma (Roth, 1964) suggesting that there is no relationship between the proliferation rate of the tumours and their RNase inhibitor content.

Acid RNase II has been found to be located in mitochondria, microsomes and lysosomes of various tissues (Koenig, 1964; Colter et al, 1961; Roth, 1963; de Lamirande, 1959). This enzyme is active over the pH range from 4.8 to 5.8 and it is resistant to inactivation by acid but it is inactivated by heat. It appears to be specific for pyrimidine 3' phosphoryl bonds and its activity is similar to that of alkaline RNase II (Razzell, 1967). However, it is not affected by the inhibitor of the alkaline RNase (Roth, 1959).

Phosphodiesterase I, which resembles snake venom phosphodiesterase, is heat labile, optimally active at pH 9.2 and requires a divalent cation

such as $Mg^{2+}$ for maximal activity. The enzyme is located in the microsomal fraction (Razzell, 1961). Chains with a terminal 3' phosphate group are relatively resistant to the enzyme which is very active against chains with a 5' phosphate terminal group. The enzyme releases 5' mononucleotides stepwise from the 3' hydroxyl end (Razzell, 1961). In contrast to exo-RNase I, it is not specific for RNA since it will hydrolyze RNA, DNA, partially degraded polynucleotides, dinucleotides and some synthetic nucleotide esters.

Phosphodiesterase II activity has been located in the microsomal fraction of the spleen and in the supernatant fraction from homogenates of various tissues (Razzell, 1961). The enzyme is also heat labile but is optimally active at pH 6. This hydrolase releases 3' mononucleotides from the 5'hydroxyl end of the chain but 5' phosphate terminated oligonucleotides are resistant (Razzell and Khorana, 1961). As was the case with phosphodiesterase I, this enzyme is relatively non-specific.

More recently, a 3' exo-RNase located on the surface of Novikoff hepatoma ascites cells was reported to be responsible for the degradation of polynucleotides which adsorbed to the cells. The enzyme is not located intracellularly and is not released into the extracellular fluid (Crooke et al, 1972). An RNase which impaired the infectivity of encephalomyocarditis virus has also been found on the surface of Krebs ascites cells (Bases and Huppert, 1966). These surface RNases may play an important role in protecting cells from exogenous nucleic acids.

## Possible Roles of RNA-Degrading Enzymes in RNA Metabolism

It is well established that several species of functional RNA such as rRNA and mRNA arise from large molecular weight precursors

(Scherrer et al, 1963; Darnell, 1968; Weinberg, 1973). These rapidly labelled precursors include pre-rRNA in the nucleolus and the Hn-RNA in the nucleoplasm. Both these precursors are processed in the nucleus and the mature RNA is then transported to the cytoplasm. This processing or degradation of high molecular weight RNA must involve the action of RNases but the precise mechanism of the RNase activity is not fully understood.

Both 28S and 18S rRNA are derived from a common 45S precursor by a series of cleavages (Weinberg and Penman, 1970). After methylation of the 45S rRNA precursor, the two methylated regions are cleaved from the non-methylated regions and become the rRNA. The non-methylated regions (approximately half of the 45S RNA) are discarded and this portion seems to be highly unstable since attempts to isolate it have failed (Weinberg and Penman, 1970). A third cleavage product of the rRNA precursor molecule appears to be a 7S RNA which is hydrogen-bonded to the 28S RNA (Pene et al, 1968). The only species of rRNA which does not seem to involve processing is 5S RNA (Darnell, 1968).

There have been several attempts to identify the RNase involved in the processing of rRNA precursors. The in vitro studies by Kelly and Perry (1971) on the digestion of nucleolar intermediates by RNases suggested that the 45S rRNA precursor was cleaved by a specific endo-ribonuclease followed by limited trimming with an exo-RNase which requires $Mg^{2+}$ and releases 5' monocleotides. The endo- and exo-RNase I reported by Lazarus and Sporn (1967) may be involved in this process. Mirault and Scherrer (1972) also concluded from their studies that an endo-nuclease is involved in the processing of pre-rRNA in the nucleolus of HeLa cells. In the nuclei of Novikoff hepatoma cells an RNase is closely associated with ribonucleoprotein particles which has a higher affinity

for 45S RNA than mature 28S and 18S RNA (Prestayko et al, 1972 and 1973). This enzyme shares many properties with the alkaline RNase II although it seems to be more heat labile. An inhibitor of this enzyme was separated by DEAE-cellulose chromatography. How the cell controls the specific cleavage of the pre-rRNA is not clear. Giudice et al(1973) showed that processing of pre-rRNA could be mimicked by incubating purified RNA-protein particles with pancreatic RNase and suggested that the protein associated with the RNA likely protects certain regions of the RNA from degradation.

The synthesis of mRNA must also involve specific nucleolytic cleavage. The Hn-RNA, which is generally believed to be the precursor of mRNA, represents 1 - 3% of the total RNA. The life time of Hn-RNA is 5 to 10 minutes and therefore its turnover is very rapid (Weinberg, 1973). Only a small portion of Hn-RNA is utilized for mRNA synthesis while the remainder is degraded (Houssais and Attardi, 1966; Darnell, 1968). However, there is still no indication which enzymes are involved in this process.

A further example where RNases must function specifically is in the processing of tRNA. In mammalian cells, the 4S tRNA is synthesized in the nucleus and it immediately appears in the cytoplasm as a poly-nucleotide about 10 - 20 bases longer than the mature tRNA (Kay and Cooper, 1969). Over a period of 15 minutes, the tRNA is cleaved, methylated and rare bases are formed. The tRNA processing is apparently mediated by enzymes in the cytoplasm (Culp and Brown, 1968).

Other possible functions of the RNases are the intracellular digestion and turnover of RNA such as mRNA. These functions are probably carried out, in part, by acid nucleases located in the lysosomes.

Under normal conditions, the lysosomal enzymes hydrolyze, intravesicularly, waste materials or foreign materials in the cells. The compartmentation of these enzymes probably serves to regulate their activities thus rendering them harmless towards cellular nucleic acids engaged in protein synthesis (de Duve, 1963).

Functional RNA may also become resistant to the RNase by forming a complex with proteins. For example, in intact polysomes, rRNA is unaffected by levels of RNase which readily attacks ribosomal subunits (Utsunomiya and Roth, 1966). It seems that the control of rRNA turnover can be dependent on other factors, for example, low $Mg^{2+}$ concentration which dissociates ribosomes, rather than upon regulation of a specific RNase. The control of RNase activity can also be exerted by the endogenous RNase inhibitors as single stranded mRNA was shown to be protected by the inhibitor of alkaline RNase II.

## The Uptake of Exogenous RNA by Ehrlich Ascites Cells

A number of mammalian cells have been shown to take up homologous and heterologous RNA. This subject was reviewed by Bhargava and Shanmugam (1971). In order to determine the amount of RNA taken up by the cells, $^{3}H$, $^{14}C$ or $^{32}P$ labelled RNA has been used and the radioactivity associated with the cells after incubation has been determined directly or after autoradiography. The amount of RNA actually ingested is questionable in many investigations because of the possibility of adsorption of RNA to the cell surface. In some cases RNase has been used to destroy the surface-bound RNA. While about 30% of the total radioactivity associated with spleen and lymph node cells was removeable

by the RNase treatment (Chin and Silverman, 1967), very little was removeable from Nelson's ascites cells (Yoon, 1965). Part of the RNA taken up can be located in the nuclei (Galand and Ledoux, 1966; Schwarz and Reiche, 1962). Studies by Galand et al (1966) also showed that the RNA taken up was not lost during incubation in the absence of RNA. Niu et al (1968) showed in their autoradiographic studies that at least a portion of the RNA associated with the Nelson's ascites cells was taken up intracellularly.

Earlier studies on the uptake of RNA did not specify the nature of the exogenous RNA. The RNA was generally labelled with radioactive precursors, extracted with phenol and then incubated with the cells. It is therefore likely that such preparations contained high molecular weight as well as low molecular weight RNA. Even in a recent study in which the exogenous RNA was described as almost completely acid insoluble, the gel electrophoresis profile of the RNA suggested that it contained heterogenous high molecular weight RNA and also RNA smaller than 4 - 5S (Juliano and Mayhew, 1972). Herrera et al (1970), however, isolated tRNA from Escherichia coli which had amino acid acceptor activity and demonstrated its uptake by murine leukemia and human lymphoblast cells lines. Few studies have been undertaken to determine whether certain classes of RNA are taken up preferentially.

Except in a few cases (Herrera et al, 1970; Schell, 1971), the uptake experiments were carried out without assaying for RNase activities in the medium or monitoring the nature of the extracellular RNA during incubation. It seems likely that degradation of RNA can take place extracellularly unless special precautions are used since RNase activity cannot be eliminated from the incubation medium. However, nucleotides

and larger products of RNase digestion were not taken up by cells (Holoubek et al, 1966) and it has been suggested that degraded RNA is not a likely source of radioactivity incorporated from exogenous RNA.

Although completely depolymerized RNA is not taken up (Bishop and Abramhoff, 1966), mammalian cells can take up a variety of RNAs and there does not seem to be any specificity with respect to the uptake of homologous or heterologous RNA (Shanmugam and Bhargava, 1966), or to the size of the RNA. Furthermore, RNA of different sizes can compete with the exogenous RNA for uptake (Schell, 1971). The amount of RNA taken up is dependent on the concentration of RNA but there is wide disagreement on whether higher concentrations of RNA increase (Colby and Chamberlin, 1969) or decrease uptake (Chin and Silverman, 1967). It is possible that there is an optimum concentration of RNA at which RNA uptake is greatest and increasing RNA concentration would therefore initially increase and then decrease uptake.

The mechanism by which RNA is taken up is not clear but many reports suggest that pinocytosis might be responsible. However, poly-L-lysine which does not affect pinocytosis (Cohn and Parks, 1966), can sharply increase the uptake of RNA by Ehrlich ascites cells (Mayhew and Juliano, 1973). Furthermore, metabolic inhibitors such as 2,4-dinitrophenol and iodoacetate which inhibit pinocytosis have been reported to have variable effects on the uptake of RNA (Juliano and Mayhew, 1972; Shanmugam and Bhargava, 1966). In addition to poly-L-lysine, several agents have been demonstrated to increase the uptake of RNA. Protamine sulfate stimulated the uptake of E.coli RNA by chick embryo cells (Amos and Kearns, 1963), and Cohn and Parks (1966) suggested that it stimulates uptake by protecting the RNA from RNase and/or by stimulating

pinocytosis. This stimulatory effect of protamine sulfate, however, was not reproducible in a similar experiment with Nelson's ascites cells (Yoon, 1965). DEAE-dextran had also been reported to increase the uptake of RNA (Colby and Chamberlin, 1969). One report, however, suggested that DEAE-dextran was responsible for the increased binding of poly I : poly C to the surface of fibroblasts (Bausek and Merigan, 1969).

The exposure of mammalian cells to exogenous RNA appears to have various effects on the recipient cells. Several studies have indicated that exogenous heterologous RNA stimulates synthesis of a protein which usually is not synthesized or synthesized at a very low rate. For example, liver RNA had been shown to stimulate synthesis of serum albumin, tryptophan pyrrolase and glucose-6-phosphatase in Nelson's and Ehrlich ascites cells (Niu et al, 1961; Niu, 1963). These workers proposed that the proteins are synthesized in response to the information contained in the RNA taken up since degradation of the RNA prior to its uptake destroyed the activity. Furthermore, ascites cells can synthesize a species-specific serum albumin after taking up a fraction of liver RNA believed to be rich in mRNA (Niu et al, 1972). Niu's claim that tumour cells which do not ordinarily synthesize serum albumin, tryptophan pyrrolase and glucose-6-phosphatase synthesize these proteins after they have been treated with liver RNA was not reproducible by Imsand and Ephrussi (1964). This raised some doubts as to whether intact RNA was the cause of the promotion of protein synthesis. However, the translation of foreign mRNA in recipient cells as proposed by Niu et al is not inconceivable in view of the finding by other workers that the RNA was not degraded 40 minutes to 2 hours after uptake (Galand et al, 1966; Galand and Ledoux, 1966). Colby and Chamberlin (1969) suggested that part of

the RNA was degraded only after 4 - 24 hours of incubation and the degradation products were then incorporated into host RNA. It is clear from these studies that RNA is taken up and degraded intracellularly. What enzymes are responsible for degradation of ingested RNA and how these enzymes are controlled is not known.

The stability of the ingested RNA seems to depend on the cell type involved (Niu, 1963; Colby and Chamberlin, 1969; Galand et al, 1966; Schell, 1971). By determining the stability of the ingested RNA, some insight might be gained with respect to the role of intracellular RNases. Since many RNases may be involved, little information can be gained by studying the fate of ingested RNA in one type of cell. However, if the fate of the RNA taken up by two or more cell types which differ in RNase activities can be compared, then the differences in the rate of degradation of the ingested RNA, perhaps, can be accounted for by the differences in the RNase activities between these cells.

Ehrlich ascites cells can be obtained with normal and high RNase activities. Increased RNase activity had been demonstrated in Ehrlich ascites cells after in vitro treatment of the cells before transplantation with several cytostatic agents, including actinomycin D (Erbe et al, 1966). In a more recent study, 7 days after the actinomycin D treatment, the activity of phosphodiesterase II was increased 2.5 fold, alkaline RNase II 7.2 fold and acid RNase II 10 fold accompanied by a decrease in the RNase inhibitor to undetectable levels (von Tigerstrom, 1972). Although the cause of these activity changes is not certain, cells with high RNase activities would be expected to degrade RNA faster than cells with normal RNase activities.

Therefore in this project, the uptake of bacterial rRNA and the

fate of the ingested RNA in Ehrlich ascites cells with normal and high RNase activities were compared. Labelled bacterial rRNA isolated from Pseudomonas aeruginosa was characterized and incubated with the two cell types and the fate of the ingested RNA was determined. The uptake of low molecular weight bacterial RNA and extensively degraded RNA was also studied. It was hoped that by comparing the fate of the ingested RNA in the two types of Ehrlich ascites cells, some understanding could be gained with respect to the physiological functions of acid RNase II, alkaline RNase II and the endogenous RNase inhibitor.

# MATERIALS AND METHODS

## Materials

Uracil-2-$^{14}C$, 54.8 mCi/mmole, and uridine-2-$^{14}C$, 59.8 mCi/mmole, were purchased from the New England Nuclear Corporation. Actinomycin D and highly polymerized yeast RNA were products of Calbiochem. Polyadenylic acid was purchased from Miles Laboratories Inc. Bio-Gel P-60, 100 - 200 mesh, was obtained from Bio-Rad Laboratories, DNase from P-L Biochemical Inc. and RNase A from the Worthington Corporation. Acrylamide and N,N'-methylene-bisacrylamide were products of Eastman Organic Chemicals and were purified according to Loening (1967). "Fischer's medium for Leukemic cells in mice" was obtained from the Grand Island Biological Company. All other chemicals were of reagent grade and were obtained commercially. The solutions and buffers used in this investigation are as follows:

| | | |
|---|---|---|
| Solution 1 | NaCl | 0.14 M |
| | $CaCl_2$ | 0.0022 M |
| | KCl | 0.0047 M |
| | $NaPO_4$ | 0.004 M, pH 7.0 |
| Solution 2 | NaCl | 0.14 M |
| | Tris-HCl | 0.01 M |
| | Glucose | 1 mg/ml |
| | $NaPO_4$ | 0.004 M, pH 7.4 |
| Solution 3 | NaCl | 0.05 M |
| | $MgCl_2$ | 0.0015 M |
| | 2-mercaptoethanol | 0.01 M |
| | Tris-HCl | 0.01 M, pH7.4 |
| Solution 4 | NaCl | 0.15 M |
| | NaEDTA | 0.01 M, pH 7.4 |
| | Tris-HCl | 0.01 M, pH 7.4 |
| Tyrode Solution | NaCl | 0.14 M |
| | KCl | 0.0025 M, $NaH_2PO_4$ 0.0003 M |
| | $CaCl_2$ | 0.0034 M, $NaHCO_3$ 0.012 M |
| | $MgCl_2$ | 0.0005 M, Glucose 0.1%, pH 7.7 |

| | |
|---|---|
| Solution 5 | As Tyrode Solution, but $NaCO_3$ was omitted, pH 5.4. |
| "Modified" Fischer's Medium | $NaHCO_3$ in Fischer's Medium was replaced by 0.05 M Hepes, pH 7.4. |

## Methods

### Maintenance and Collection of Ehrlich Ascites Cells

To maintain the cells, they were transplanted at weekly intervals. The cells were obtained from a mouse aseptically, suspended in solution 1, centrifuged at 1,600 x g for 1 minute and resuspended in solution 1 to obtain a 10% cell suspension. Each mouse was then injected intraperitoneally with 0.2 ml of the cell suspension (5 - 6 x $10^6$ cells). For some experiments the tumour cells (2.5 - 3 x $10^7$ cells/ml in solution 1) were treated with actinomycin D (5 μg/ml final concentration) for 15 minutes at room temperature before transplantation and then 0.5 ml of the cell suspension was injected per mouse. The stock solution of actinomycin D (200 μg/ml) used for this treatment was prepared in sterile 0.85% NaCl. Control animals received 0.2 ml of cells which were treated with an equivalent volume of sterile 0.85% NaCl for 15 minutes at room temperature. Tumour bearing mice were either obtained from the University of Alberta Cancer Research Unit or from the same cell line maintained in this laboratory.

The mice were killed by cervical dislocation 6 - 7 days after transplantation and the cells were collected by aspiration. The intraperitoneal cavity was washed with 8 - 10 ml of solution 2 containing 0.2 mg/ml heparin. Cell yields were estimated after centrifugation in the International Clinical Centrifuge at 1,600 x g for 1 minute. To remove traces of blood cells and ascites fluid, cells from each mouse

were washed three to six times by centrifugation (1 minute at 750 x g) and resuspension with 10 - 12 ml of solution 2. They were then packed by centrifugation for 7 minutes at 1,600 x g. The collection and washing of the tumour cells was carried out at room temperature.

Enzyme Assays

The procedure for RNase assays were essentially those described by Razzell (1963 and 1967) and by von Tigerstrom (1972). Extracts of Ehrlich ascites cells were used for these assays. Packed cells were suspended in 8 volumes of solution 3 and disrupted at 0° for 20 seconds by a Sonifier at maximum output (Bronwill Scientific). After this treatment, enzyme assays were carried out at 37°. Necessary dilutions were made in solution 3. For each assay 4 time points were taken within 20 minutes.

Alkaline and Acid RNase II

In order to determine the activity of these enzymes it was necessary to treat the cell extracts with acid and with heat to inactivate interfering enzymes and the RNase inhibitor. In the acid treatment, cell extracts (2 ml) were treated with M $H_2SO_4$ (0.4 ml) at 0° for 1 hour. Molar tris acetate (0.1 ml), pH 7.4 was added and the final pH of the solution was adjusted to 5.7 with M NaOH. The sample was then centrifuged for 10 minutes at 27,000 x g. The supernatant is the "acid-treated extract". The acid treatment destroys the RNase I activity, phosphodiesterase activities and other interfering enzymes but not acid and alkaline RNase II activities or all the alkaline RNase II inhibitor.

In the heat treatment, 2 ml of "acid-treated extract" was adjusted to pH 3.5 with M $H_2SO_4$ and heated to 60° for 22 minutes. The

pH was then readjusted to 5.7 with M NaOH and the sample was centrifuged at 27,000 x g for 10 minutes. The supernatant is the "acid- and heat-treated extract". Acid RNase II activity and the remaining inhibitor for alkaline RNase II are destroyed by this heat treatment but alkaline RNase II is resistant to heat.

Alkaline RNase II activity was assayed in 0.08 ml of 16 mM $KPO_4$, 18 mM Tris acetate, 1 mg/ml RNA, 1 mM $Na_2EDTA$, pH 7.8 using 20 µl of the prepared extracts. The same solution adjusted to pH 5.5 with acetic acid was used for the assay of acid RNase II. Both, the "acid-treated extract" and the "acid- and heat-treated extract" were assayed for acid and alkaline RNase II activities. The activity present in the "acid- and heat-treated extract" at pH 5.5 is that of alkaline RNase II and was subtracted from the activity obtained with the "acid-treated extract" in order to determine acid RNase II activity in the sample.

All assays were carried out in Beckman microfuge tubes and the reactions were stopped by the addition of 5 µl of 60% PCA. The tubes were placed in a ice bath for at least 15 minutes and then centrifuged for 30 seconds in a Beckman microfuge. Fifty µl of the supernatant was diluted into 0.25 ml of 4% PCA and the absorbance was determined at 260 nm.

One unit of enzyme is defined as the amount which hydrolyzes 1 µmol of substrate per minute or, in the case of ribonucleases, produces 1 µmol of acid-soluble nucleotides per minute. Specific activities are based on E 11,000 for acid-soluble products from RNA and one ml of sonicate.

Assay for Alkaline RNase II Inhibitor

The method for determining the RNase inhibitor was described by

Shortman (1961) and modified by von Tigerstrom (1972). This assay is based on the ability of the RNase inhibitor to inhibit commercial RNase A preparations. The cell extract was centrifuged at 27,000 x g for 30 minutes. Varying amounts of the supernatant solution, usually diluted with solution 3, were added to a constant amount of RNase A ($3.3 \times 10^{-3}$ µg/ml in 0.5 ml solution 3 prepared fresh from 1 mg/ml RNase A in 0.1% gelatin). Twenty µl of this mixture was then assayed for alkaline RNase II activity. One unit of inhibitor is the amount which inhibits $3.3 \times 10^{-3}$ µg RNase A by 50%.

Preparation of $^{14}$C-Labelled Pseudomonas aeruginosa rRNA

Pseudomonas aeruginosa ATCC 9027 was grown at 37° with shaking in a medium containing $NH_4H_2PO_4$ (0.3%), $K_2HPO_4$ (0.2%), glucose (1%), $MgSO_4 \cdot 7H_2O$ (0.1%) and $FeSO_4$ (0.5 ppm) adjusted to pH 7.2 before sterilization. Glucose and $MgSO_4$ were added separately after sterilization. To initiate growth of the organism, a 10 - 15% inoculum of a 16 hour culture was added to fresh medium. The turbidity of the culture at 600 nm was determined and 30 to 120 minutes after inoculation, uracil-2-$^{14}$C was added (0.1 - 2.5 µCi/ml final concentration). To measure the incorporation of uracil-2-$^{14}$C into RNA, duplicate samples of the culture (0.2 ml) were precipitated in 1 ml cold PCA (4%) and the precipitates were washed on Millipore filters (HA = 0.45 µm) with 11 ml 4% cold PCA and a final wash with 2 ml of 5% TCA. The filter discs were counted in a liquid scintillation counter using Bray's solution (Bray, 1960).

After 4 - 5 hours, at which time the organism had grown through 2 - 3 generations, the cells were harvested by centrifugation at 12,000 x g for 10 minutes. RNA was then extracted from the cells as described below.

Preparation of $^{14}C$-Labelled Ehrlich Ascites Cell RNA

In order to label the mammalian RNA, uridine-2-$^{14}C$ (2.5 $\mu$Ci/ml) was added to a 5% Ehrlich ascites cell suspension in "modified" Fischer's medium. After 1 hour incubation at 37° the cells were diluted five fold with "modified" Fischer's medium and incubation was continued for 6 hours in the presence of streptomycin (50 $\mu$g/ml) and penicillin (50 units/ml). The cells were harvested by centrifugation at 1,6000 x g for 5 minutes and RNA was extracted from the cells.

Extraction of RNA

The method of Penman (1966), modified by von Tigerstrom (1973), was used to extract RNA from Ehrlich ascites cells and P. aeruginosa. Washed Ehrlich ascites cells, usually from 0.5 ml of a 5% cell suspension, or P. aeruginosa from a 25 ml culture, were suspended in 4 ml of solution 4 and lysed by the addition of 0.1 ml 25% SDS and 4 ml phenol. In some experiments, extractions were carried out in the presence of bentonite (20 mg/extraction). The lysed cells were treated twice with phenol and chloroform containing 1% isoamyl alcohol (4 ml each), then 3 times with 4 ml chloroform. The extractions were carried out for 6 minutes with intermittent mixing at 55° or at room temperature. The phases were separated after each treatment by centrifugation at 1,500 x g for 10 minutes and the organic phase was removed by aspiration. The RNA was precipitated from the aqueous phase at -20° for 2 hours after the addition of 0.8 ml M sodium acetate, pH 5.0, 0.04 ml 0.1 M $MgCl_2$, and 12 ml 95% ethanol. Excess alcohol was removed under reduced pressure and the pellet was then dissolved in 1 ml 25 mM ammonium acetate, 5 mM $MgCl_2$, pH 5.5 and treated with 50 $\mu$g DNase for 30 minutes at room temperature.

The RNA was re-precipitated at -20° for at least 2 hours after the addition of 0.1 ml M sodium acetate, pH 5.5, and 3.0 ml of 95% ethanol. The final pellet was dissolved either in cold, sterile solution 5 for the preparation of high molecular weight RNA, or in 0.1 ml of electrophoresis buffer containing 10 - 15% sucrose.

Preparation of High and Low Molecular Weight RNA

Bio-Gel P-60, 100 - 200 mesh, was allowed to swell in water and the gel was washed repeatedly with water and solution 5 to remove fines. A column (1.2 cm x 40 cm ) of Bio-Gel P-60 was then prepared and washed. The washing and elution procedures were carried out in the cold with solution 5. RNA extracted from 25 ml of P. aeruginosa, dissolved in 0.5 ml of solution 5 was applied to the column and eluted in five minute or ten minute fractions (1.0 - 1.5 ml each). The initial fractions containing high radioactivities were pooled, diluted to about 14 $A_{260nm}$/ml and stored at -70°.

In some experiments, low and high molecular weight RNA were separated by their different solubilities in M NaCl (Crestfield et al, 1955). The NaCl-insoluble fraction (high molecular weight RNA) was separated from the NaCl-soluble fraction (low molecular weight RNA) by centrifugation at 12,000 x g for 5 minutes. High molecular weight RNA was then purified by Bio-Gel P-60 column chromatography as described.

The RNA soluble in M NaCl was precipitated at -20° for 120 minutes in 95% ethanol. The supernatant was removed and this soluble RNA was dissolved in cold 0.1 M Tris-HCl, pH 7.5 and applied to a DEAE-cellulose column (2.3 cm x 15 cm) equilibrated with 0.1 M Tris-HCl, pH 7.5. The RNA was eluted with M NaCl and 0.4 ml fractions were collected. The

fractions containing high radioactivities were pooled and precipitated at -20° for 120 minutes in 3 volumes of cold ethanol. Excess ethanol was removed under reduced pressure and the pellet was then dissolved in solution 5 and stored at -70°. All procedures described above were carried out in the cold with sterilized solutions.

The Measurement of RNA Uptake by Ehrlich Ascites Cells

A 5% Ehrlich ascites cell suspension was prepared in "modified" Fischer's medium and the cells were incubated at 37° with shaking. Labelled RNA was added to a final concentration of 60 µg/ml or as indicated in the text. A sample of the cell suspension (0.05 - 0.1 ml) was added to Bray's solution directly to determine the total radioactivity present. At zero time or as soon as samples can be taken (0 - 10 seconds) and at timed intervals, 0.35 - 0.5 ml of the cell suspension were removed and immediately suspended in 10 ml solution 5 or Tyrode solution. The cells were centrifuged at 1,000 x g for 3 minutes in a PR-J Centrifuge or at 1,600 x g for 3 minutes in a Clinical Centrifuge. The supernatant was discarded and the pellet resuspended in 10 ml of solution 5. This washing procedure was repeated 3 to 4 times at 4° or at room temperature. After the final wash, the cell pellet was suspended in 0.5 ml of solution 5 and the radioactivity associated with the cells was counted in Bray's solution. Any remaining cells were removed by 3 rinses with solution 5 and counted.

To determine the acid-insoluble radioactivity associated with the cells, aliquots of 0.2 - 0.3 ml of the cell suspensions were removed and washed 3 to 4 times in solution 5 as described above. The washed cells were then suspended in 0.5 ml of solution 5 and precipitated in 4% PCA for at least 20 minutes. The precipitated samples were applied

to Whatman No. 3 filter papers and washed with a total of 11 ml of cold 4% PCA and finally with 2 ml of cold 5% TCA. The uptake of RNA was expressed as the amount of radioactive RNA or as a percentage of total radioactivity associated with the cells.

Electrophoresis of RNA

Polyacrylamide gel electrophoresis was carried out in a 2% gel containing 0.5% agarose as described by Peacock and Dingman (1968) with some modifications. To prepare 20 ml of gel, the following components were combined at 40°: 6.76 ml $H_2O$, 1 ml 0.5 M sodium phosphate, pH 7.2, 2 ml polyacrylamide-bisacrylamide (20% : 1%), 0.12 ml 10% TEMED, 10 ml 1% agarose and 0.12 ml 10% ammonium persulfate. The gels were cast in 5 mm or 7 mm i.d. glass tubing and pre-run in 50 mM sodium phosphate, 1 mM EDTA, 0.1% SDS, pH 7.2 (electrophoresis buffer) to equilibrate and to remove UV absorbing material. RNA extracted from 2.5 ml of 5% Ehrlich ascites cells was applied to 7 mm gels and separated for 130 minutes at room temperature using 5 mA per gel. For gels cast in 5 mm tubes, samples were applied and separated for 75 - 80 minutes at room temperature using also 5 mA per gel. The gels (9.2 cm) were scanned at 260 nm using a Gilford model 240 spectrophotometer connected to a Photovolt recorder. After scanning, the gels were cut into approximately 2 mm slices to determine the radioactivity in the gels. The slices were incubated with 0.2 ml of concentrated $NH_4OH$ for 18 hours at 37° and counted in a liquid scintillation counter after addition of Bray's solution.

To determine the molecular size of "degraded" RNA, polyacrylamide gel electrophoresis was carried out in 7.5% gels. To prepare 10 ml of gel, the following components were combined at room temperature: 5.65 ml

$H_2O$, 3.75 ml acrylamide-bisacrylamide (20% : 0.5%), 0.5 ml sodium phosphate, pH 7.2, 0.05 ml TEMED and 0.05 ml sodium persulfate. Samples were applied to gels in 5 mm i.d. glass tubing and separated for 70 - 75 minutes at room temperature using 5 mA per gel. The absorbance and the radioactivity concentration was determined as described above.

Preparation of Crude Inhibitor

Ehrlich ascites cells were suspended in 8 volumes of 1 mM EDTA, 1 mM DTT, 50 mM $KPO_4$, pH 6.0 and disrupted by sonication for 20 seconds at 0° using a Sonifier. The cell extract was centrifuged at 27,000 x g for 30 minutes. Ammonium sulfate was added to the supernatant with stirring at 0° and the precipitate obtained between 35% and 50% saturation with ammonium sulfate was dissolved in the above solution and dialyzed for 120 minutes in solution 3. The RNase inhibitor activity and also the RNase I activity was determined.

# RESULTS

## Preparation and Characterization of Radioactive Bacterial Ribosomal RNA

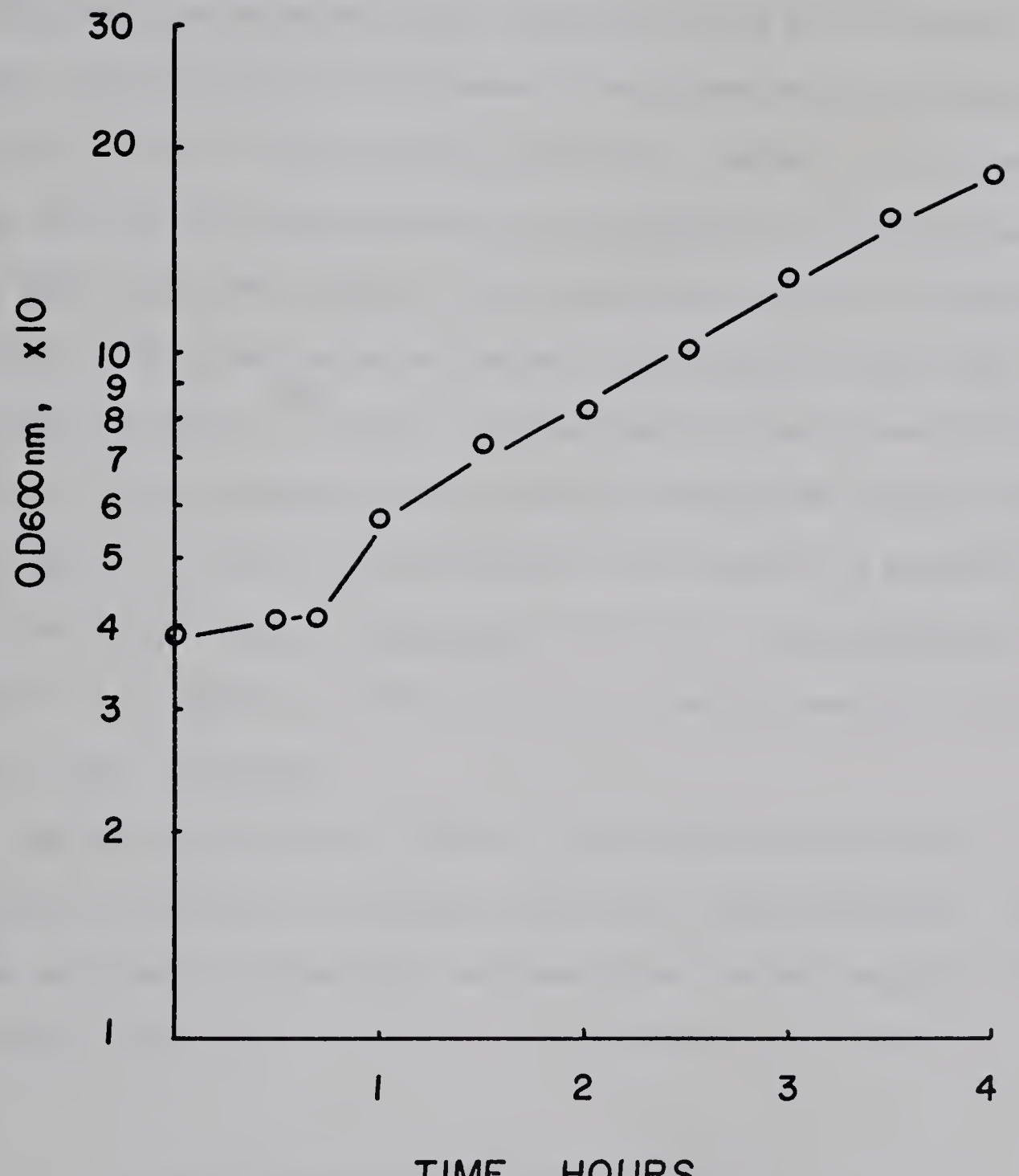
Bacterial ribosomal RNA was chosen as the exogenous RNA for studies of the uptake and metabolism of RNA by Ehrlich ascites cells for two reasons. The first was that $^{14}C$ labelled bacterial ribosomal RNA can be prepared in good yield with high specific activities. Secondly, the bacterial rRNA separates from mammalian rRNA when it is analyzed by polyacrylamide gel electrophoresis and thus it can be distinguished from the host RNA even if its degradations products are used for biosynthesis by Ehrlich ascites cells.

For the preparation of labelled RNA of high specific activity from P. aeruginosa, it was essential to use a radioactive precursor which is incorporated into RNA with a high efficiency. Uracil-2-$^{14}C$ was found to be satisfactory for the labelling of this P. aeruginosa RNA. Shortly after inoculation, $^{14}C$-uracil was added to give a final concentration of 0.1, 0.5, 1.0 or 2.5 $\mu$Ci/ml. As shown in Fig. 1, the organism grew in the glucose - inorganic salts medium with a generation time of approximately 1.5 hours. The initial and final cell densities, measured spectrophotometrically at 600 nm, were approximately 0.2 - 0.4 and 2.0 respectively. To determine the amount of uracil incorporated into acid precipitable material at various times during growth, samples of the culture were removed, acid precipitated and washed as indicated in Methods. Between 50% and 70% of the radioactivity was incorporated into acid-insoluble material after 30 minutes. Maximum incorporation of

FIGURE 1

GROWTH OF PSEUDOMONAS AERUGINOSA

P. aeruginosa was grown at 37° with shaking in an inorganic salts medium containing glucose as carbon and energy source. The medium was inoculated to an optical density of 0.2 to 0.4 with an overnight culture. Uracil-2-$^{14}$C (0.5 μCi/ml) was added 40 minutes after inoculation. Cell growth as measured by turbidity at 600 nm could reach values as high as 2.2 to 2.8 but cells were normally harvested at a value of about 2.


30
20
10
9
8
7
6
5
4
3
2
1
OD600nm, x10
1
2
3
4
TIME, HOURS

radioactivity into rRNA and uniform labelling were ensured by allowing the cells to grow about 2 generations in the presence of $^{14}$C-uracil.

After extraction with phenol and chloroform and digestion with DNase, low molecular weight labelled RNA was removed by gel filtration as shown in Fig. 2. Between 73% and 96% of the $^{14}$C-uracil incorporated into acid-precipitable material was recovered by the RNA extraction procedure. The elution profile showed a large peak which was eluted within the initial 15 fractions (15 to 30 ml). The RNA in this large peak was analyzed by polyacrylamide gel electrophoresis and was found to contain 85% to 92% rRNA (Fig. 3). The subsequent fractions eluted from the Bio-Gel P-60 column contained largely low molecular weight RNA. Between 30% and 45% of $^{14}$C-uracil incorporated into acid precipitable material could be recovered as high molecular weight RNA. Cells labelled with 0.1 $\mu$Ci/ml $^{14}$C-uracil were found to yield rRNA with a specific activity of 0.6 $\mu$Ci/mg and a $A_{260}/A_{280}$ ratio of 2. Increasing the $^{14}$C-uracil to 2.5 $\mu$Ci/ml in the culture increased the specific activity of RNA to about 13 $\mu$Ci/mg.

The RNA was stored at -70° and to determine its stability, it was analyzed periodically by polyacrylamide gel electrophoresis. The profiles obtained from these gel indicated that the RNA remained stable for at least 2 months.

## Uptake of RNA by Ehrlich Ascites Cells

Before the degradation of ingested RNA in Ehrlich ascites cells could be studied, it was necessary to establish methods for determining the amount of high molecular weight RNA taken up by these cells under

FIGURE 2

PURIFICATION OF BACTERIAL rRNA BY CHROMATOGRAPHY ON BIO-GEL P-60

A 300 ml culture of P. aeruginosa was labelled with 0.1 μCi/ml uracil-2-$^{14}$C. The RNA was extracted, dissolved in sterile solution 5 and applied to a 1.2 x 45 cm Bio-Gel column. The RNA was eluted with sterile solution 5 as indicated in Methods. Fractions of 1.1 ml each were collected at a flow rate of 0.2 ml/min. Ten μl were removed from each fraction and used to determine the radioactivity. Fractions 8 - 13 were pooled and the RNA in these fractions was found to have a specific activity of 0.77 μCi/mg.

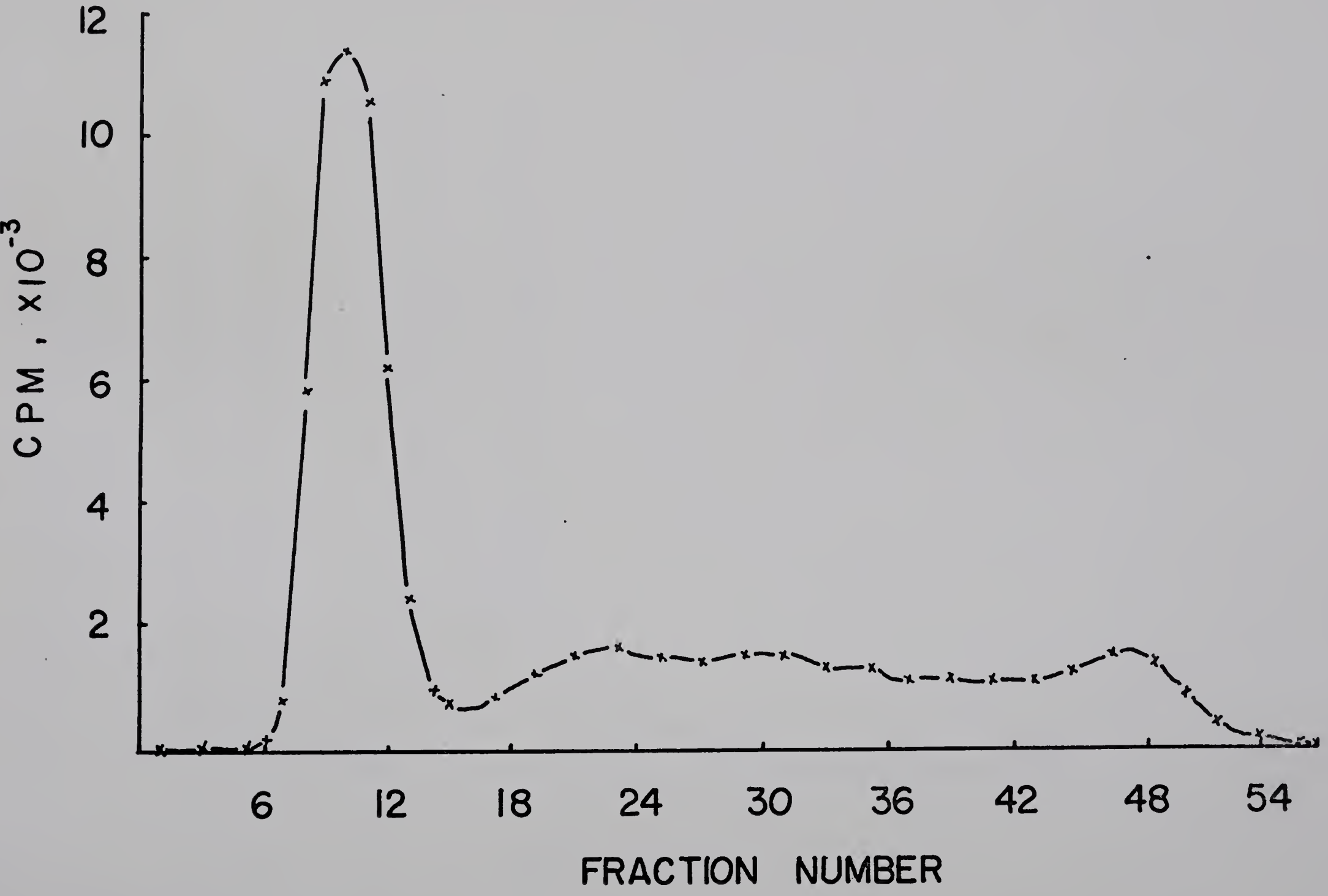
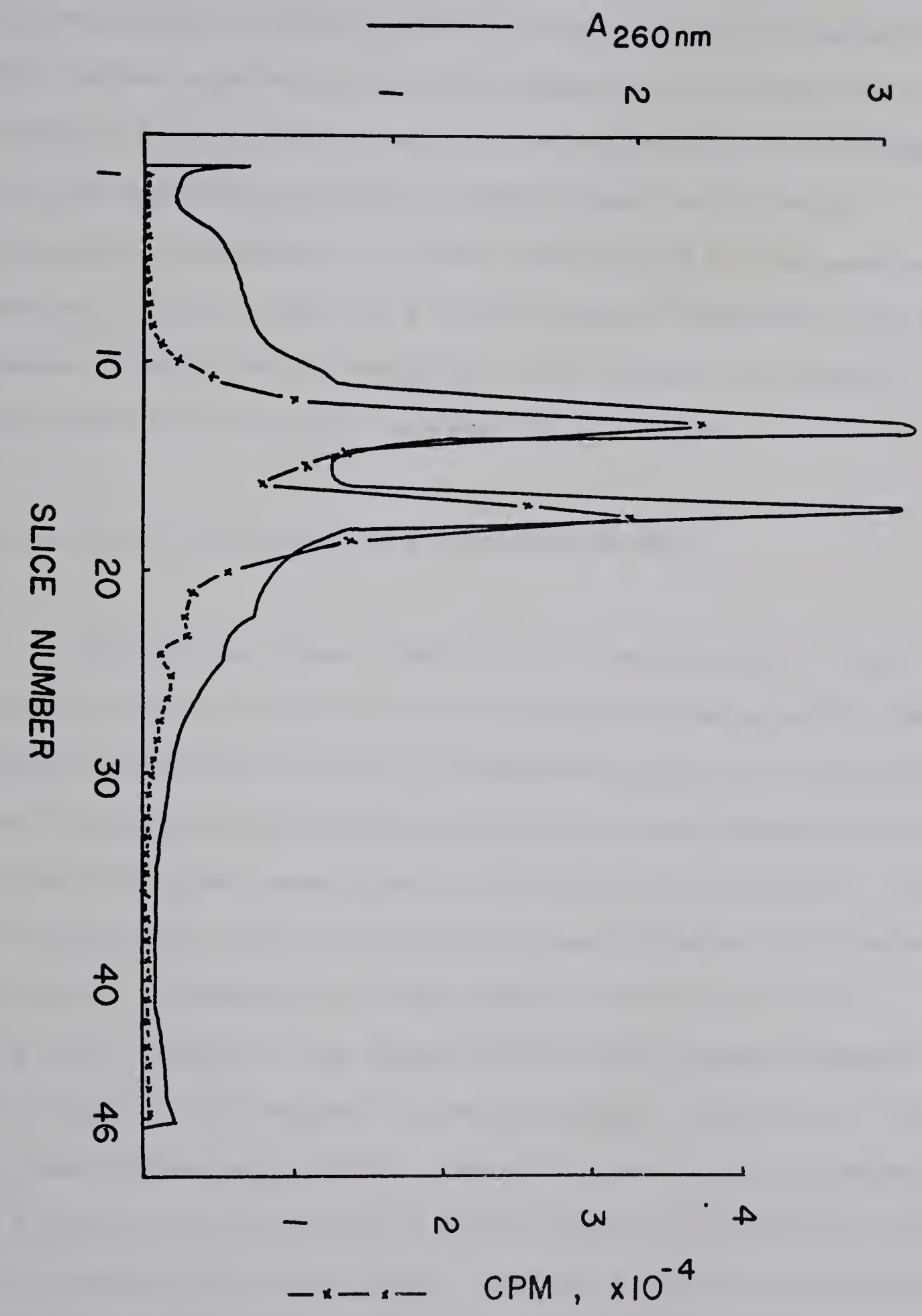

CPM , X10-3
12
10
8
6
4
2
6
12
18
24
30
36
42
48
54
FRACTION NUMBER

FIGURE 3

POLYACRYLAMIDE GEL ELECTROPHORESIS OF
BACTERIAL RIBOSOMAL RNA

Labelled RNA (0.06 mg) which had been purified by Bio-Gel P-60 column chromatography was separated in a 2% polyacrylamide gel at 5 mA for 85 minutes. The gel was scanned at 260 nm (——) and the radioactivity in the gel slices was determined (-×-). The specific activity of the RNA was 5.5 μCi/mg.


A 260 nm
1
2
3
1
10
20
30
40
46
SLICE NUMBER
1
2
3
4
CPM , x10^-4

different conditions. Some of the factors which might affect the amount of RNA taken up are cell concentrations, types of incubation medium, RNA concentrations and the use of DEAE-dextran which was reported to facilitate uptake of RNA by chick embryo cells (Colby and Chamberlin, 1969). Uptake experiments were also conducted in the presence of actinomycin D because during later studies on the stability of ingested RNA it was desirable to inhibit the RNA synthesis while RNA was incorporated. Furthermore, since the rRNA prepared for the experiments contained 8 - 15% non-rRNA and a certain amount of degradation could be expected to take place extracellularly, the uptake of low molecular weight RNA and of extensively degraded RNA was studied.

Determination of RNA Uptake by Ehrlich Ascites Cells

To determine uptake of RNA by Ehrlich ascites cells, it was decided to measure the total amount of radioactive exogenous RNA that remained associated with the cells because the fate of this RNA could later be traced. Several methods of removing excess RNA that was not incorporated or closely associated with the cells were attempted. The first method was to wash cells which had been incubated with labelled RNA directly on Whatman No. 3 filter papers or Millipore filters (pore size = 0.45 μm). Even though the cells were washed repeatedly with solution 5, the radioactivity associated with the cells on filter discs was high and very variable. When cells were washed on Whatman No. 3 filters, the filtrates were cloudy suggesting that not all cells were retained on the filter discs. Washing the cells on filter discs therefore seemed unsatisfactory for the removal of excess RNA.

Another method which was used to remove excess RNA involved washing the cells by repeated centrifugation and resuspension. Results of a typical uptake experiment using this method is shown in Fig. 4. In this experiment, Ehrlich ascites cells were incubated in solution 5 with 60 μg/ml labelled RNA. Cells were removed at the indicated times and washed by repeated centrifugation and resuspension in solution 5 as indicated in Methods. This washing procedure was repeated six times but the radioactivity found in the supernatants suggested that three to four washes were sufficient to remove excess RNA from the cells. This is in agreement with the results of Mayhew and Juliano (1973). In order to determine the amount of labelled RNA associated with the cells, the washed cells were suspended in 0.5 ml solution 5 and the radioactivity was determined in Bray's solution. The tubes were rinsed several times to recover all washed cells. It was found that the cell suspension and the first three rinses contained more than 95% of the radioactivity which was associated with the cells after the washing procedure. The number of cells counted in a hemocytometer before and after the washing procedure was identical indicating that there was essentially no cell loss during washing.

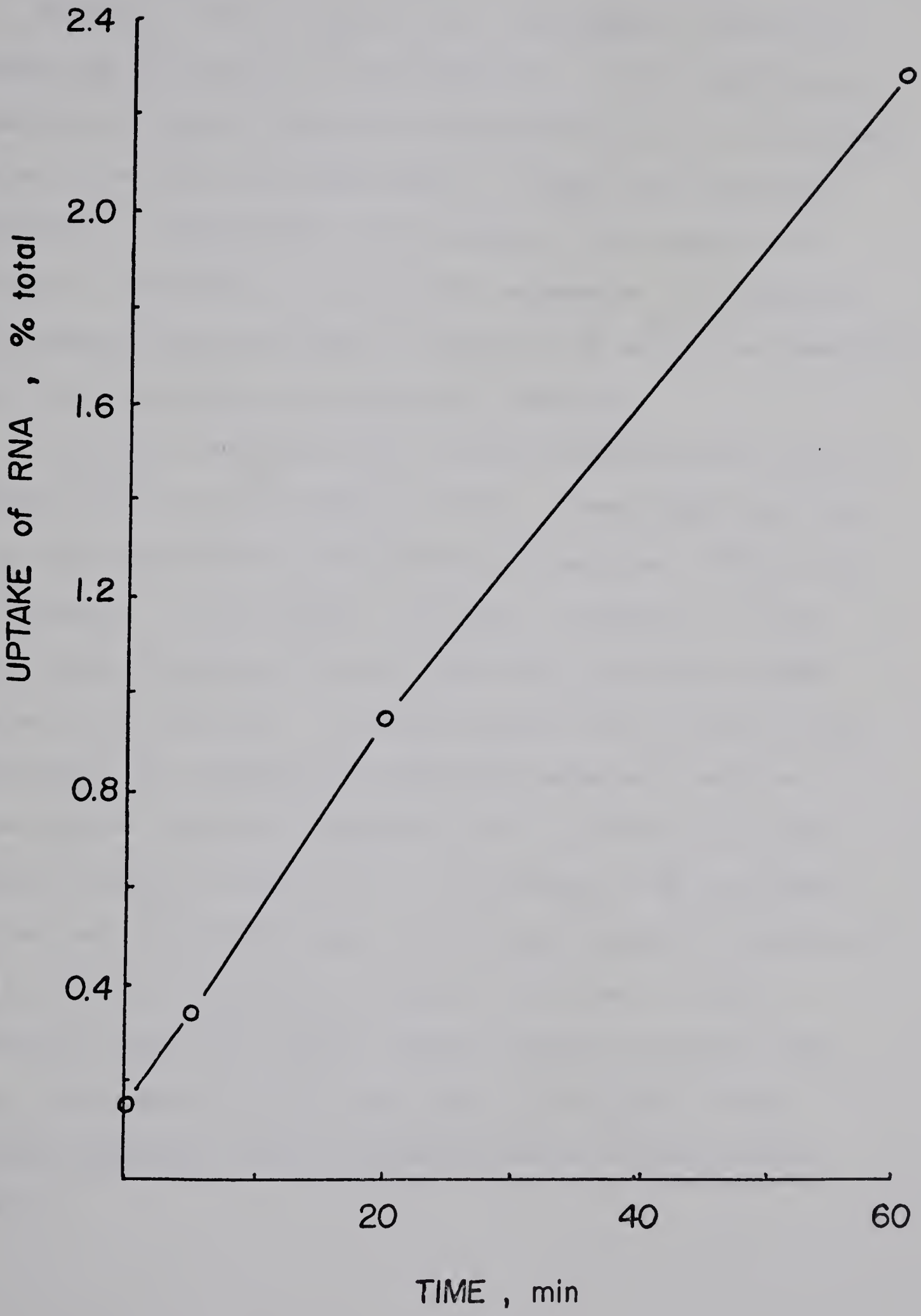
As indicated in Fig. 4, the amount of RNA taken up by the cells increased almost linearly with time and only 0.15% of the total RNA in the medium was associated with the cells at zero time. Most of this RNA was probably adsorbed to the cell surface but the amount was not great enough to cause concern.

Therefore, this method seemed to be very effective in removing excess RNA from the cells and was routinely used in determining the uptake of RNA by Ehrlich ascites cells. It is time consuming however. For example, it took 20 - 30 minutes to wash the cells 3 - 4 times and this long procedure severely limited the frequency at which samples could

FIGURE 4

UPTAKE OF RNA BY EHRLICH ASCITES CELLS

Ehrlich ascites cells were incubated in solution 5 with 60 μg/ml labelled RNA. At indicated times, the radioactivity associated with the cells was determined after repeated washing by centrifugation and resuspension in solution 5. The specific activity of the RNA was 0.37 μCi/mg.


UPTAKE of RNA , % total
2.4
2.0
1.6
1.2
0.8
0.4
20
40
60
TIME , min

be taken and it also limited the number of incubations which could be carried out in an experiment.

During the initial stages of this investigation, the washing procedure was carried out at room temperature. At this temperature, degradation of ingested RNA could be expected to go on at a significant rate while the cells were being washed. To study the intracellular degradation of ingested RNA in later studies, it was necessary to wash cells in the cold to slow down this degradation. An experiment which compared the effectiveness of washing at $4^{\circ}$ and at room temperature showed that the results were essentially identical.

Initially, washing the cells had been carried out with solution 5 (Tyrode solution without $NaHCO_3$, pH 5.4). It was thought that this low pH might contribute to precipitation or adsorption of RNA to the cell membrane. It was important, therefore, to determine to what extent the pH of washing solutions would affect the amount of RNA associated with the cells. At timed intervals after the addition of labelled RNA, cell samples were removed and washed with solution 5, Tyrode solution, solution 5 adjusted to pH 7.4 with NaOH, and with "modified" Fischer's medium, pH 7.4. The amounts of RNA associated with the cells were similar when the cells were washed with solution of pH 5.4 or 7.4, or with Tyrode solution as indicated in Figure 5. Washing with "modified" Fischer's medium resulted in slightly lower values. The reason for this is not clear. During most of the following experiments washing was carried out with Tyrode solution at pH 7.7.

The Effect of Incubation Conditions on the Uptake of RNA

Balanced salt solutions such as Tyrode solution (Yoon, 1965) or cell culture medium without serum (Juliano and Mayhew, 1972) had been used as incubation medium in various uptake experiments. As described above, initial uptake experiments were performed with solution 5 as the incubation medium. This solution was similar to Tyrode solution except $NaHCO_3$ had been omitted. Since Fischer's medium for leukemic cells in mice had been used successfully for long term incubations of Ehrlich ascites cells by Caldwell and Chan (1970) and in this laboratory (von Tigerstrom, 1972 and 1973), uptake experiments were carried out to compare the results with cells incubated in solution 5 and in "modified" Fischer's medium. Figure 6 shows that the amount of RNA associated with the cells incubated in "modified" Fischer's medium was 3 - 4 times higher than that associated with cells in solution 5. At zero time, about 0.5% of total RNA was associated with the cells incubated in "modified" Fischer's medium as compared to 0.1% in solution 5. This suggests that RNA in "modified" Fischer's medium might adsorb faster to the cell membrane than in solution 5. However, this amount is small compared to the subsequent uptake. Since RNA uptake was higher in "modified" Fischer's medium than in solution 5, experiments were routinely carried out in "modified" Fischer's medium.

Actinomycin D had been reported to have no effect on the uptake of RNA (Holoubek et al, 1966; Juliano and Mayhew, 1972). It seemed necessary, however, to verify this observation since actinomycin D was used extensively in subsequent experiments to prevent incorporation of RNA degradation products into Ehrlich ascites cell RNA. Actinomycin D at 5μg/ml inhibited the rate of incorporation of $^{14}C$-uridine by about

FIGURE 5

THE EFFECT OF DIFFERENT WASHING SOLUTIONS ON THE UPTAKE OF RNA BY EHRLICH ASCITES CELLS

Ehrlich ascites cells were incubated in "modified" Fischer's medium with 60 μg/ml rRNA. At timed intervals, cells were centrifuged and washed with Tyrode solution, pH 7.7 ( □ ), solution 5 without 0.1% $NaHCO_3$, pH 5.4 ( Δ ), solution 5 adjusted to pH 7.4 with NaOH ( O ), and "modified" Fischer's medium ( ∇ ).

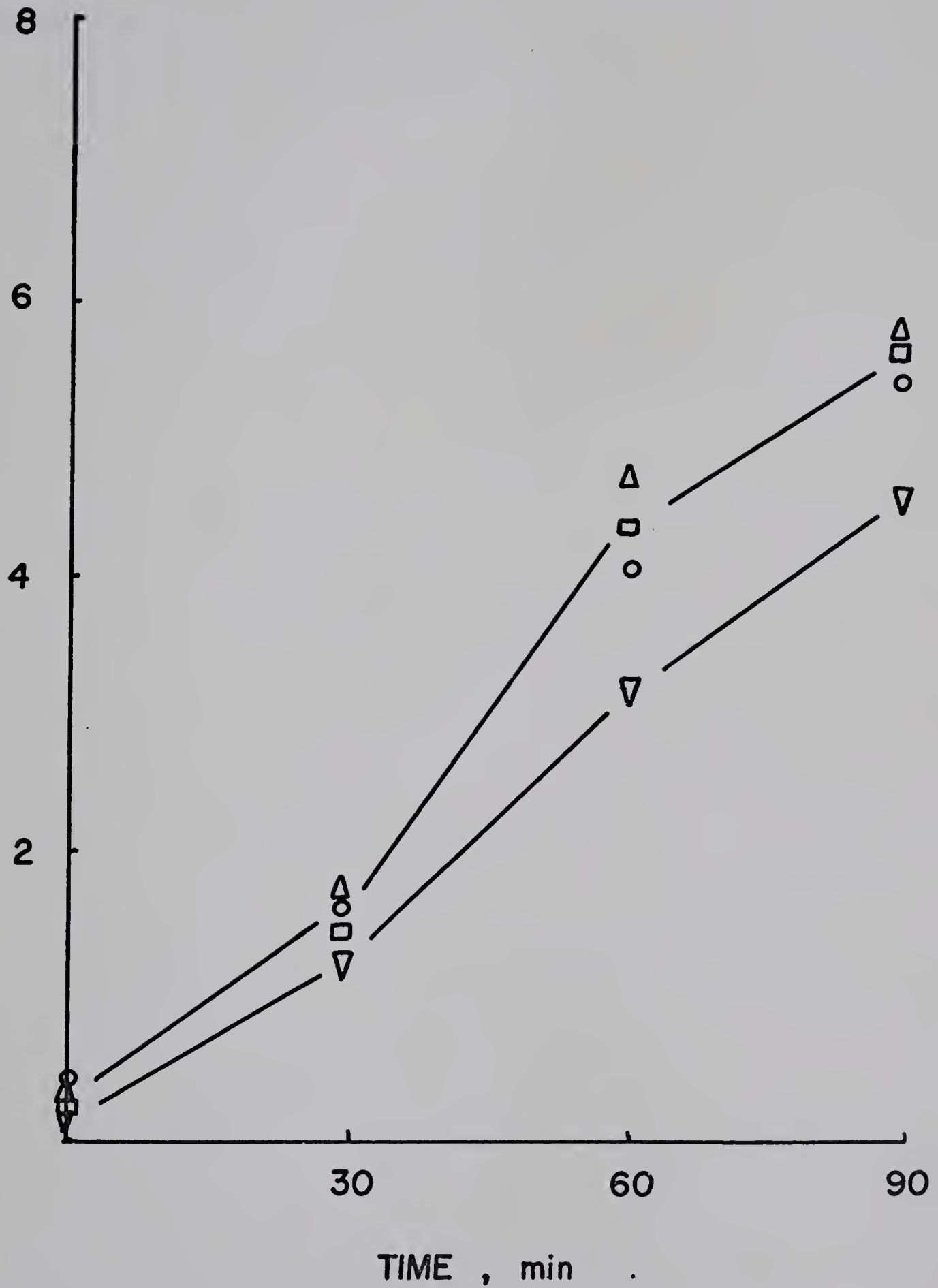
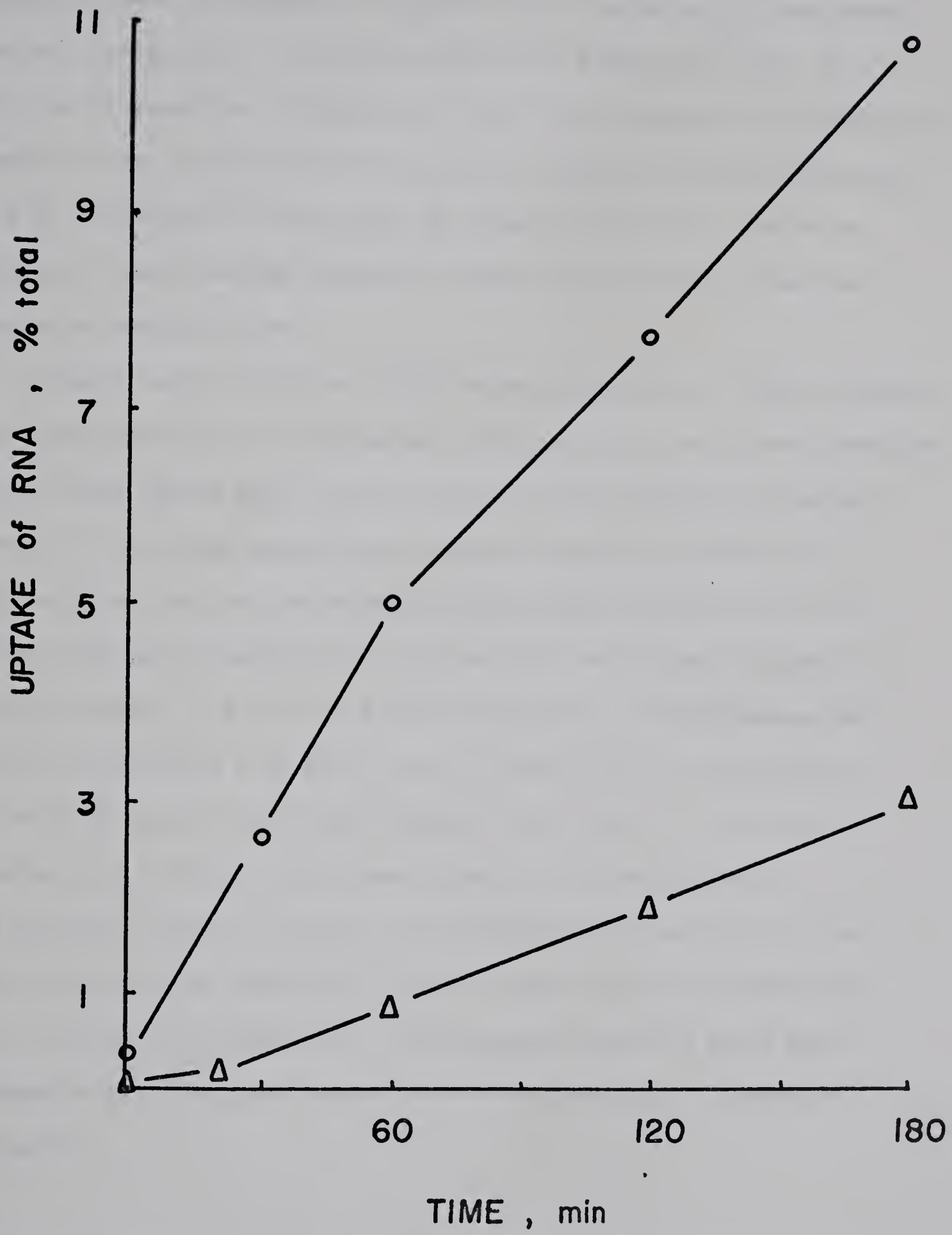

UPTAKE of RNA , % total
8
6
4
2
30
60
90
TIME , min .

FIGURE 6

UPTAKE OF RNA BY EHRLICH ASCITES CELLS INCUBATED IN "MODIFIED" FISCHER'S MEDIUM AND SOLUTION 5

Ehrlich ascites cells in "modified" Fischer's medium ( O ) and in solution 5 ( Δ ) were incubated with 60 μg/ml RNA. The amount of RNA associated with the cells after repeated washings was determined at the indicated times.


UPTAKE of RNA , % total
11
9
7
5
3
1
60
120
180
TIME , min

95% (unpublished results). To determine the effect of actinomycin D on the uptake of RNA, actinomycin D (5 μg/ml final concentration) was added to the cell suspension 2 - 5 minutes before the addition of RNA. As a control, cells were also incubated with RNA in the absence of actinomycin D. The amount of RNA associated with the cells incubated in the presence and absence of actinomycin D was similar as shown in Figure 7. Therefore, inhibition of mammalian RNA synthesis seemed to have little effect on the uptake of exogenous RNA.

Several reports (Colter, 1962; Mayhew and Juliano, 1973) indicated that damaged cells tend to take up more RNA than intact cells and therefore the rate of RNA uptake seems to be related to the viability of the cell population. Since some experiments involved long term incubation of Ehrlich ascites cells at the relatively high cell concentration of 5% it was of interest to determine their viability during long incubations. At timed intervals, 0.1 ml of the cell suspension, in the presence and absence of actinomycin D (5 μg/ml), was diluted to 0.5 ml and added to 0.1 ml of 0.4% trypan blue in buffered saline. After 5 - 15 minutes the stained and unstained cells were counted in a hemocytometer. Viable cells are known to exclude this stain whereas dead cells do not. It was found that less than 5% of the cell population was stained even after 4 - 5 hours of incubation. This suggested that the cells were not damaged after long term incubation in the presence or absence of actinomycin D.

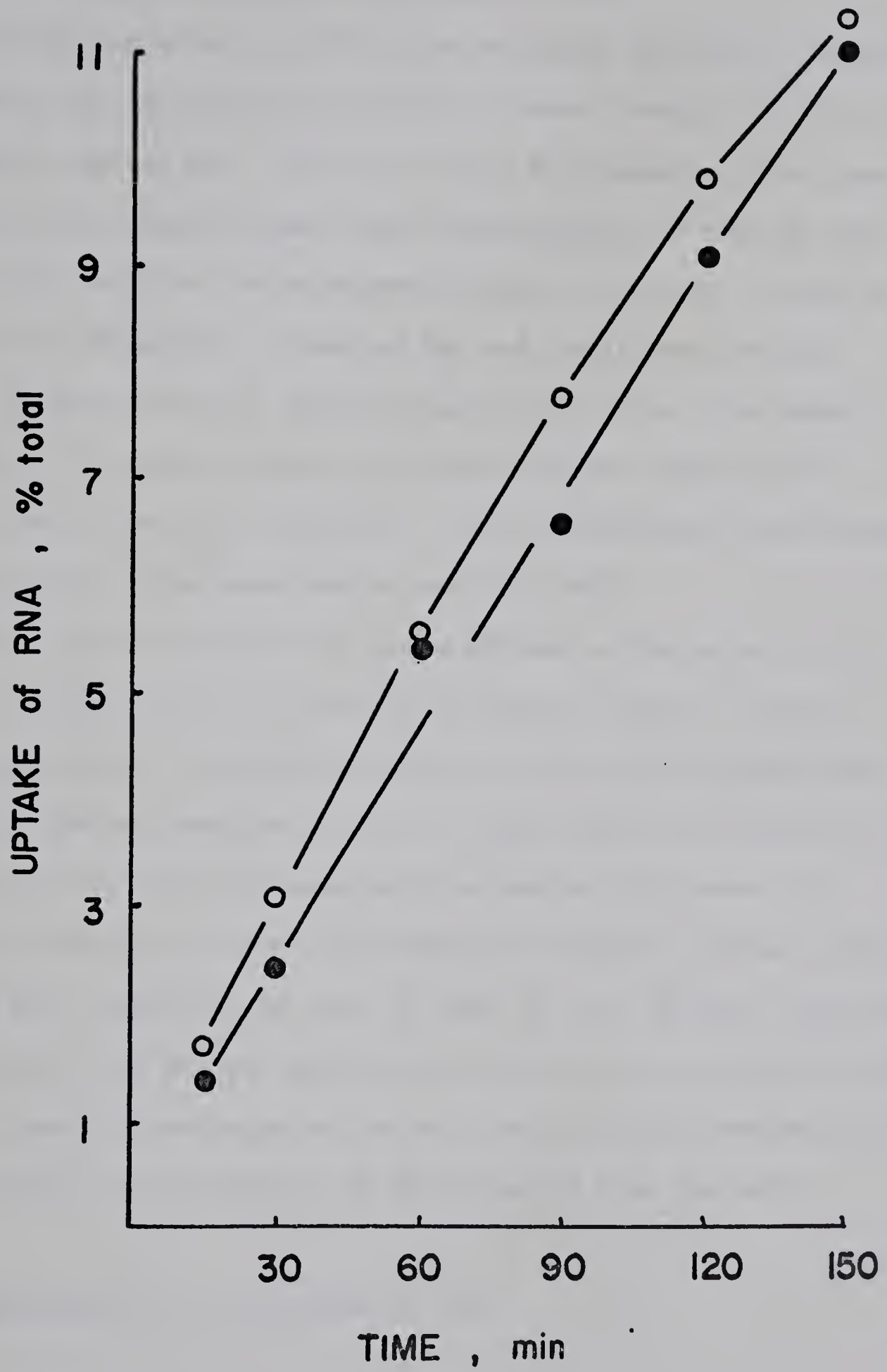
Effect of RNA Concentration and Cell Concentration on the Uptake of RNA

In attempts to increase the uptake of RNA by Ehrlich ascites

FIGURE 7

EFFECT OF ACTINOMYCIN D ON THE UPTAKE OF RNA BY EHRLICH ASCITES CELLS

Ehrlich ascites cells in "modified" Fischer's medium were incubated with 60 μg/ml RNA in the presence ( ● ) and in the absence of 5μg/ml actinomycin D ( O ). The amount of RNA associated with the cells after repeated washings was determined.


UPTAKE of RNA , % total
11
9
7
5
3
1
30
60
90
120
150
TIME , min

cells, experiments were carried out to determine the effects of varying the RNA and cell concentrations on the uptake of RNA.

To study the effect of RNA concentrations on RNA uptake, Ehrlich ascites cells were incubated in "modified" Fischer's medium with 30, 60 and 120 μg/ml labelled RNA. As shown in Fig. 8 the amount of RNA taken up per ml of cell suspension was highest when 60 μg/ml of RNA was used. To confirm this result a similar experiment was carried out in which a constant amount (60 μg/ml) of labelled RNA and, additional varying amounts of unlabelled RNA (0, 30 and 60 μg/ml) were used. The amount of RNA taken up in terms of ng/ml cell suspension was highest with 60 μg/ml of RNA in the cell suspension. This concentration was therefore routinely used for uptake experiments involving rRNA.

The effect of various cell concentrations on the uptake of RNA by Ehrlich ascites cells was studied by incubating 2.5%, 5%, 10% and 15% cell suspensions in "modified" Fischer's medium with 60 μg/ml RNA. The uptake of RNA was measured and Fig. 19 shows that the radioactivity associated with the cells increased with increasing cell numbers but the amount of RNA taken up per cell seemed to be roughly similar regardless of the cell concentration used, at least up to a 10% cell concentration. It was decided to use 5% cell suspensions since at this concentration the uptake was linear for extended periods and yet sufficient radioactivity was incorporated for the analysis of RNA extracted from the cells.

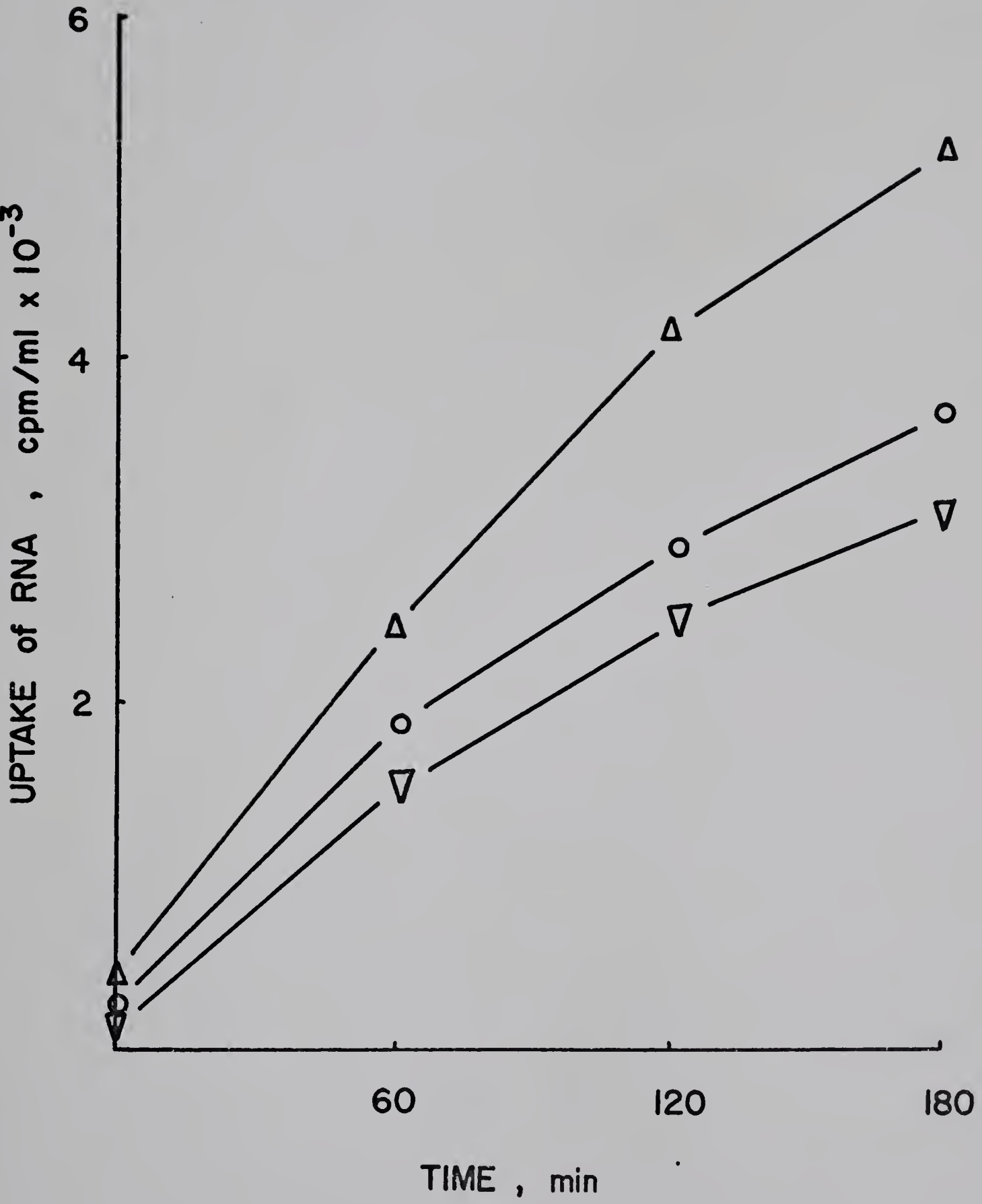
Effect of DEAE-dextran on the Uptake of RNA

An experiment was carried out to determine if the stimulatory effect of DEAE-dextran on the uptake of RNA, proposed by Colby and

FIGURE 8

UPTAKE OF RNA BY EHRLICH ASCITES CELLS IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF RADIOACTIVE RNA

Ehrlich ascites cells were incubated in "modified" Fischer's medium with 30 μg/ml ( ∇ ), 60 μg/ml ( Δ ) and 120 μg/ml ( o )of labelled RNA. The uptake is expressed as the radioactivity associated with 1 ml of cell suspension.


UPTAKE of RNA , cpm/ml x $10^{-3}$
6
4
2
0
60
120
180
TIME , min

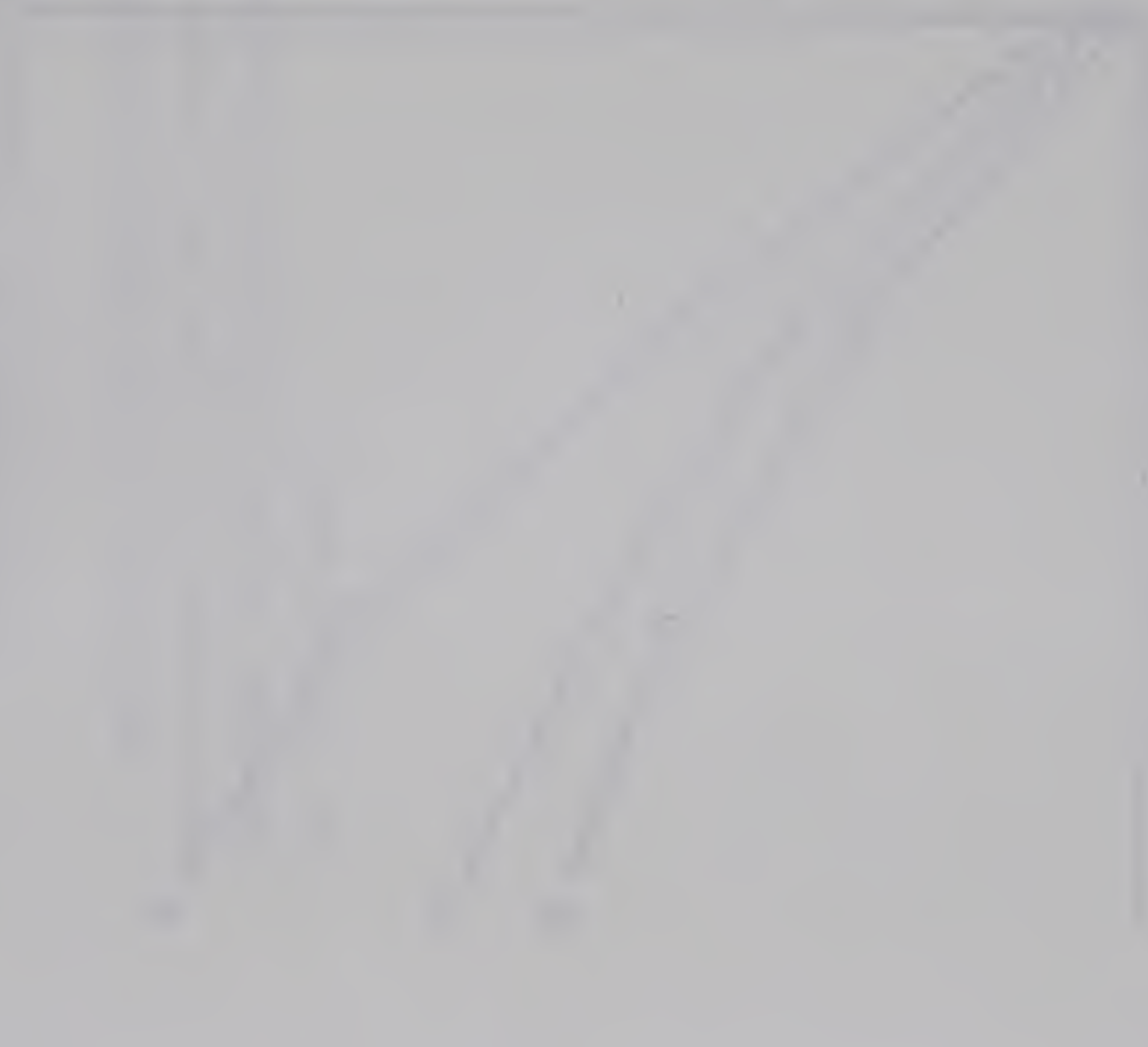
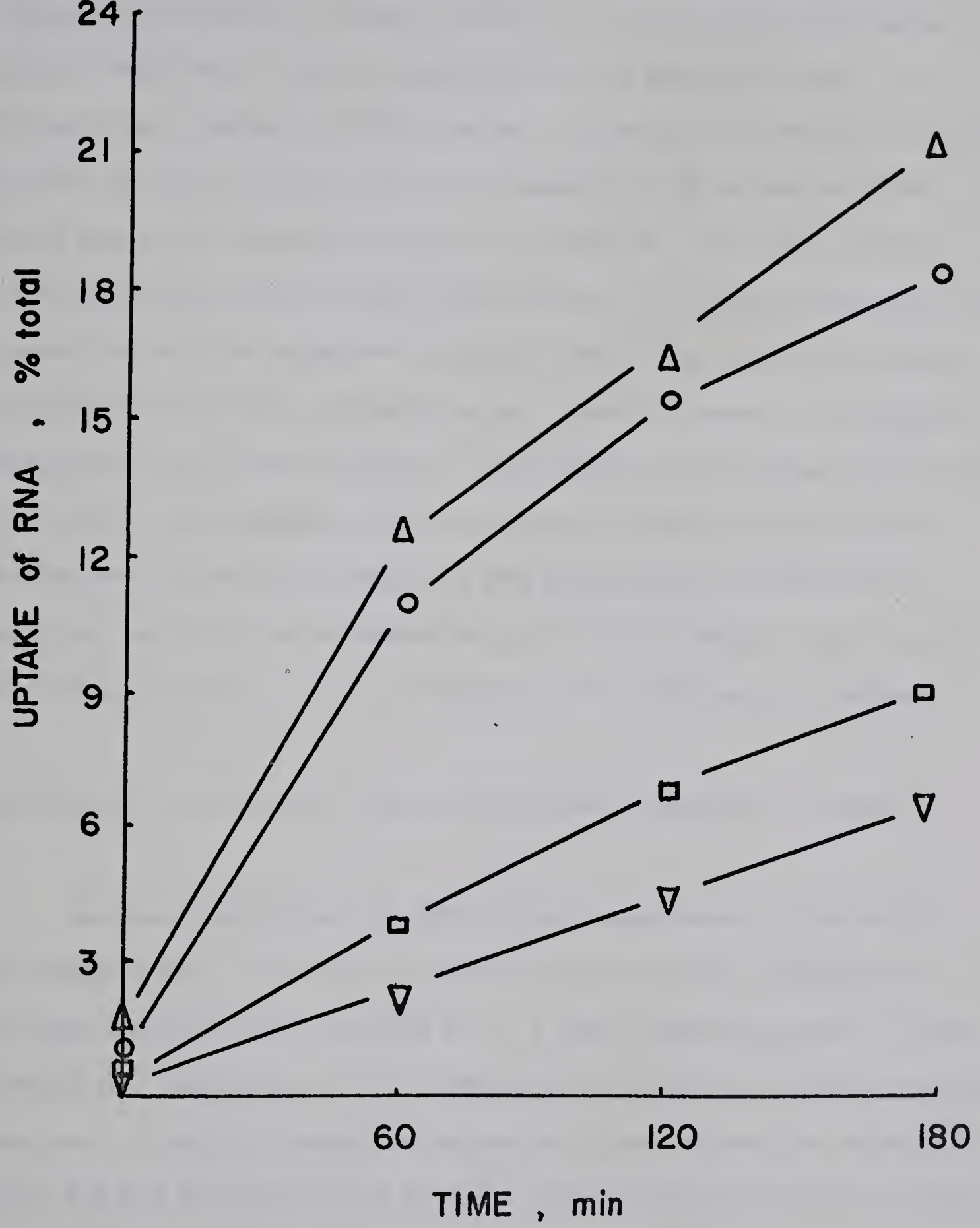


FIGURE 9

UPTAKE OF RNA BY EHRLICH ASCITES CELLS INCUBATED AT DIFFERENT CELL CONCENTRATIONS

Ehrlich ascites cells at 2.5% ( ∇ ), 5%( □ ), 10% ( o ) and 15% ( Δ ) were incubated in "modified" Fischer's medium with 60 μg/ml RNA. The specific activity of the RNA was 0.7 μCi/mg.


24
21
18
15
12
9
6
3
0
UPTAKE of RNA , % total
60
120
180
TIME , min

Chamberlin (1969), was reproducible under the chosen conditions. Ehrlich ascites cells were incubated with 0, 10 and 40 μg/ml DEAE-dextran for 30 minutes in "modified" Fischer's medium. The cells were then washed twice in "modified" Fischer's medium before the addition of RNA. If cells were not treated with DEAE-dextran, the amount of RNA associated with the cells at zero time was low and increased to 6.5% of the total RNA after 2 hours of incubation as shown in Figure 10. The cells treated with 10 μg/ml DEAE-dextran had a higher amount of RNA associated with them at zero time but the subsequent uptake of RNA did not occur at a faster rate than that with the untreated cells. Incells treated with 40 μg/ml DEAE-dextran, even higher amounts of RNA (8%) were associated with the cells at zero time. The results suggest that pre-incubation of cells with DEAE-dextran increases the amount of RNA associated with the cells at zero time, possibly due to adsorption to the cell surface. The rate of RNA uptake, however, was not increased by this DEAE-dextran treatment.

## The Uptake of Low Molecular Weight RNA and RNA Degradation Products

As described above, the RNA for most experiments contained 85 - 92% ribosomal RNA. It was also shown that only a small percentage of the total RNA was taken up during a 1 - 2 hour incubation period. Some extracellualr degradation of this rRNA during incubation could be expected. Therefore, it was of interest to determine to what extent low molecular weight RNA and RNA degradation products were ingested by Ehrlich ascites cells.

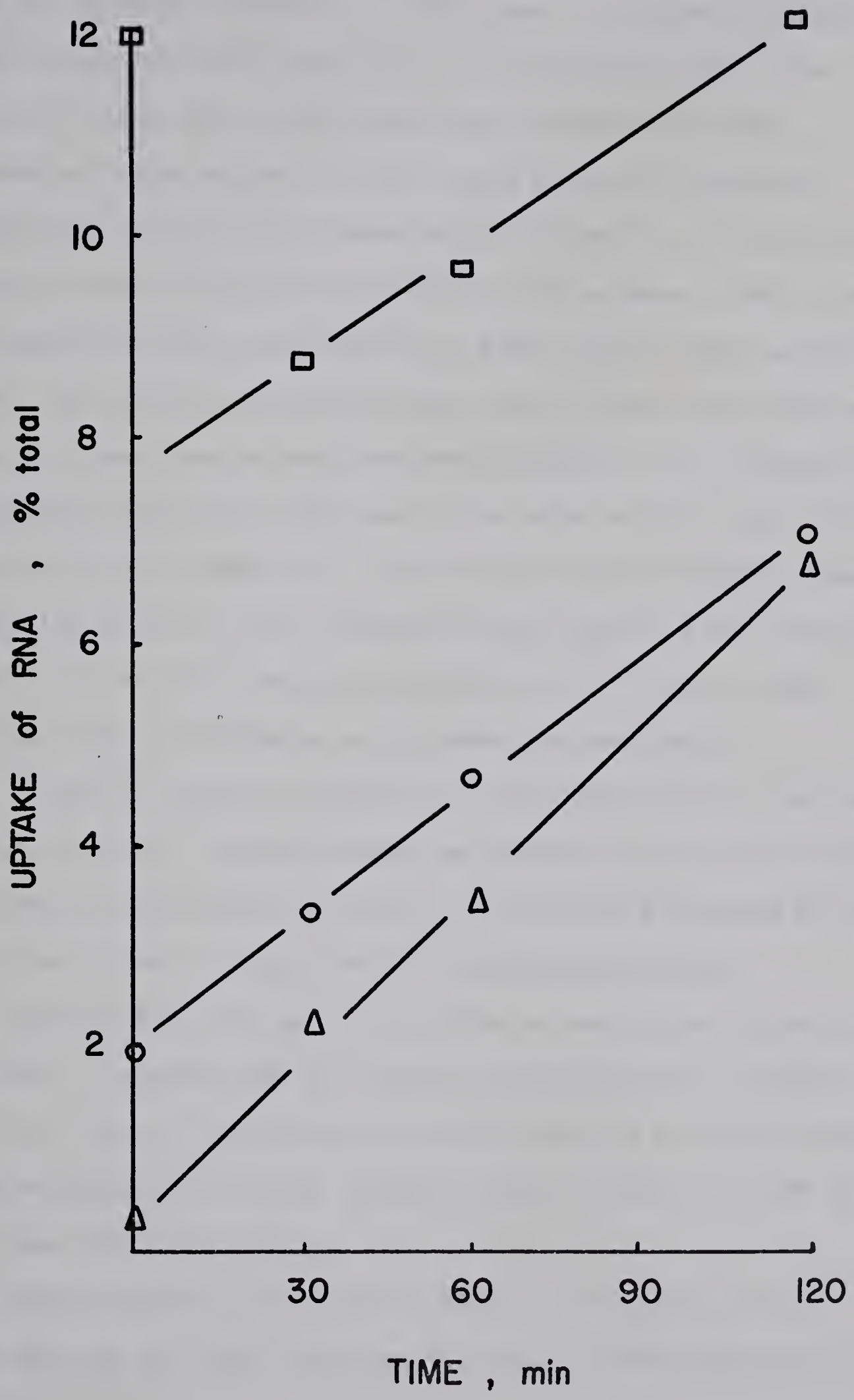
Low molecular weight RNA was prepared by fractionation with M NaCl and DEAE-cellulose chromatography as described in Methods.

FIGURE 10

UPTAKE OF RNA BY EHRLICH ASCITES CELLS

AFTER PRE-INCUBATION WITH DEAE-DEXTRAN

Ehrlich ascites cells were pre-incubated in "modified" Fischer's medium with 0 ( Δ ), 10 ( O ) and 40 μg/ml ( □ ) DEAE-dextran for 30 minutes. The cells were centrifuged, washed twice and resuspended at 5% concentration in "modified" Fischer's medium before the addition of RNA. The specific activity of the RNA was 0.7 μCi/mg


UPTAKE of RNA , % total
12
10
8
6
4
2
30
60
90
120
TIME , min

The profile of this low molecular weight RNA separated by electrophoresis in a 2% gel is shown in Figure 11. The uptake of this RNA was determined with Ehrlich ascites cells using 1.5, 6, 12 and 60 μg/ml RNA. The results also indicated that RNA uptake was dependent on the RNA concentration in the medium and total uptake as well as adsorption increased with increasing RNA concentration (Figure 12). The increase in uptake, however, was not proportional to the increase of RNA in the medium suggesting that proportionally more RNA might be taken up with less RNA. The results also indicate that low molecular weight RNA was taken up at a much reduced rate than rRNA (Figure 6). With 60 μg/ml low molecular weight RNA, uptake was in the order of 1% of total RNA after 90 minutes of incubation. Ehrlich ascites cells therefore seem to ingest low as well as high molecular weight RNA, but since the rRNA routinely used for the experiment contained only 8 - 15% non-rRNA the radioactivity attributable to its uptake was very small.

It was of interest to determine to what extent RNA of even lower molecular weight was ingested because, as pointed out below, the incubation medium contained RNase activities and some extensively degraded RNA was likely to be present in the medium as incubation progressed.

Degraded RNA was prepared and characterized before its addition to the cells. Ribosomal RNA (0.6 mg) was incubated with 2 ng RNase A at 37° for 3 hours. The profile of this RNA analyzed by polyacrylamide gel electrophoresis on 7.5% gel shows that 62% was larger than UMP but smaller than tRNA (Figure 13).

Ehrlich ascites cells were incubated with 60 μg/ml of this degraded RNA and, at timed intervals, the amount of RNA associated with

FIGURE 11

PROFILE OF LOW MOLECULAR WEIGHT RNA SEPARATED BY POLYACRYLAMIDE ELECTROPHORESIS

Low molecular weight RNA was prepared as described in Methods. About 31 μg of the low molecular weight RNA was separated in a 2% polyacrylamide gel at 5 mA for 85 minutes. The gel was scanned at 260 nm and the radioactivity of the gel slices was also measured. The specific activity of the RNA was 0.46 μCi/mg.

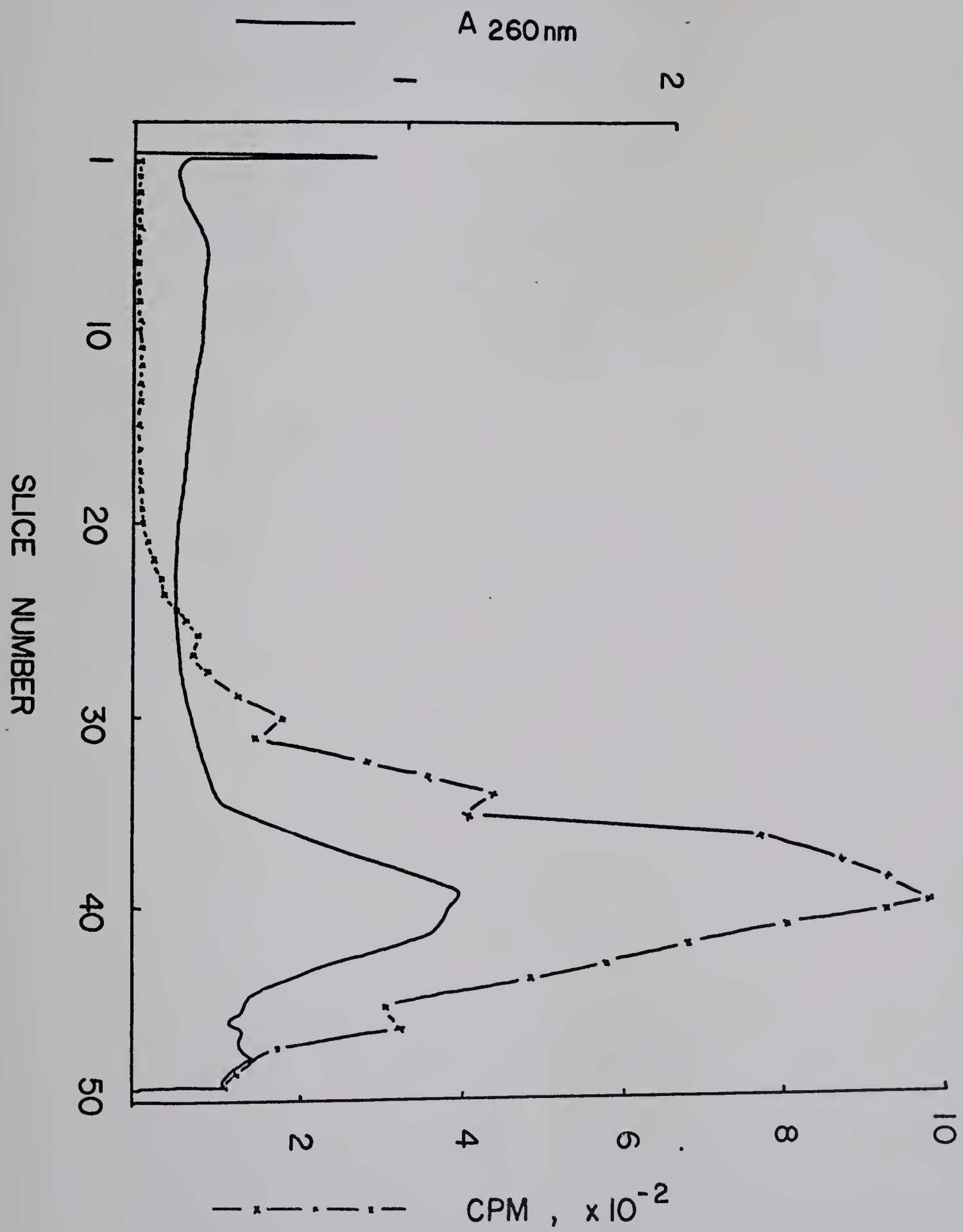

A 260nm
1
2
SLICE NUMBER
1
10
20
30
40
50
2
4
6
8
10
CPM , x 10^-2

FIGURE 12

UPTAKE OF DIFFERENT CONCENTRATIONS OF LOW MOLECULAR WEIGHT RNA BY EHRLICH ASCITES CELLS

Ehrlich ascites cells were incubated with 1.5 ( Δ ), 6 ( o ), 12 ( □ ) and 60 μg/ml ( ∇ ) of low molecular weight RNA. The uptake was expressed as the radioactivities associated with 1 ml of cell suspension.

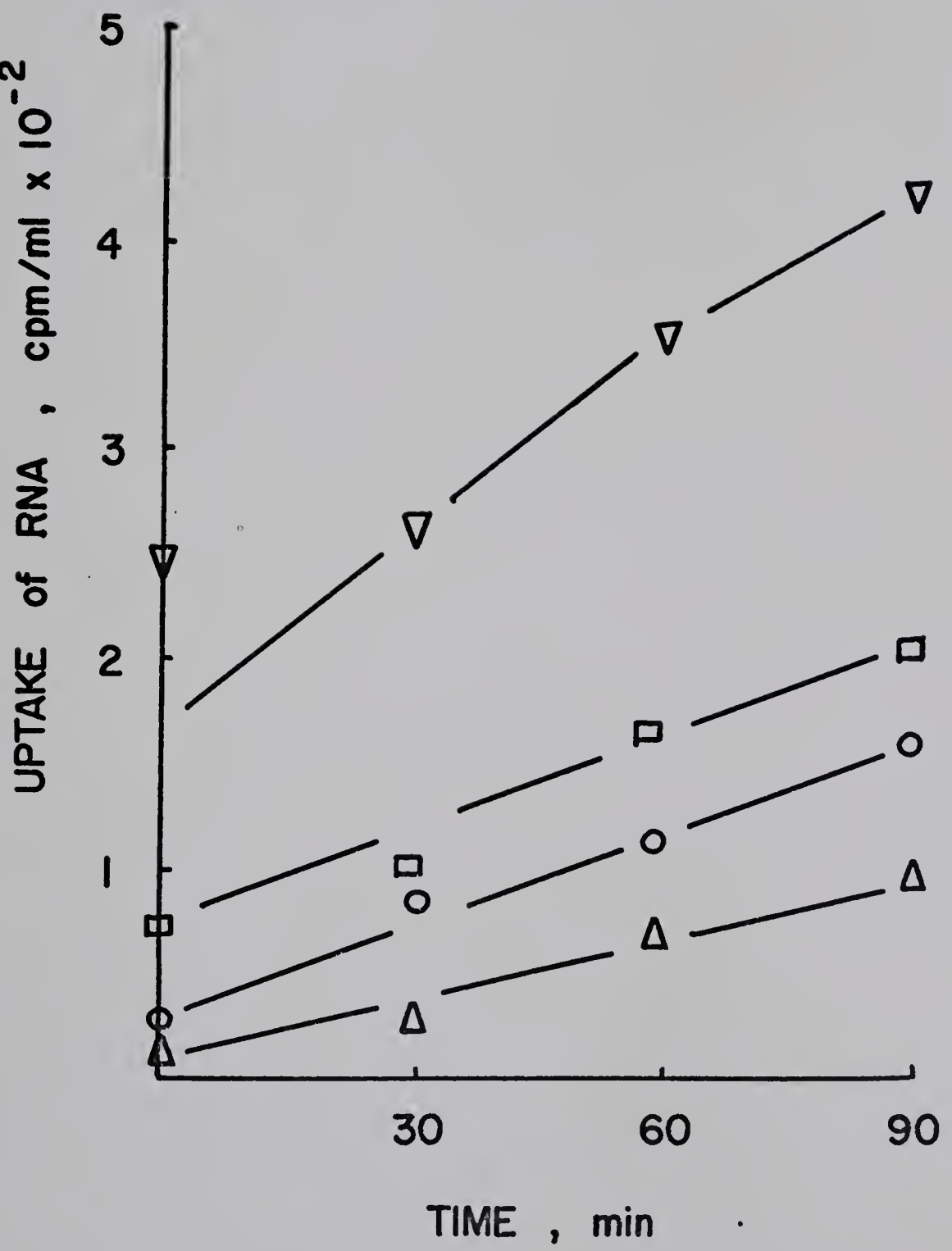

UPTAKE of RNA , cpm/ml x $10^{-2}$
5
4
3
2
1
30
60
90
TIME , min

FIGURE 13

PROFILE OF DEGRADED RNA SEPARATED BY POLYACRYLAMIDE ELECTROPHORESIS

RNA was degraded by RNase A as described in Methods and 6 μg was applied to a 7.5% polyacrylamide gel and separated at 5 mA for 75 minutes. UMP (55 μg) and tRNA (40 μg) were used as standards. The gel was scanned at 260 nm and the radioactivity of the gel slices was also measured. The specific activity of the RNA was 0.3 μCi/mg.

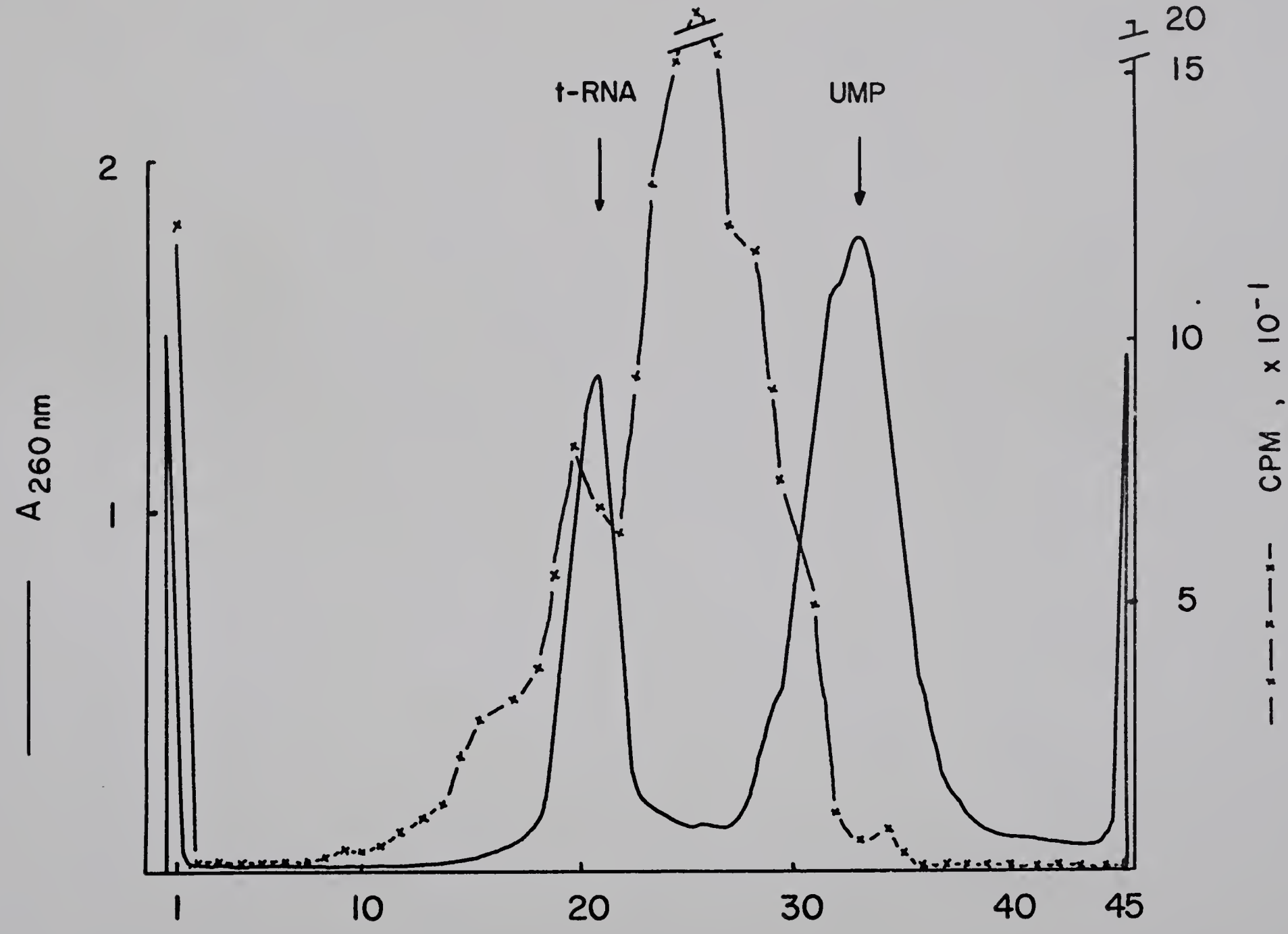
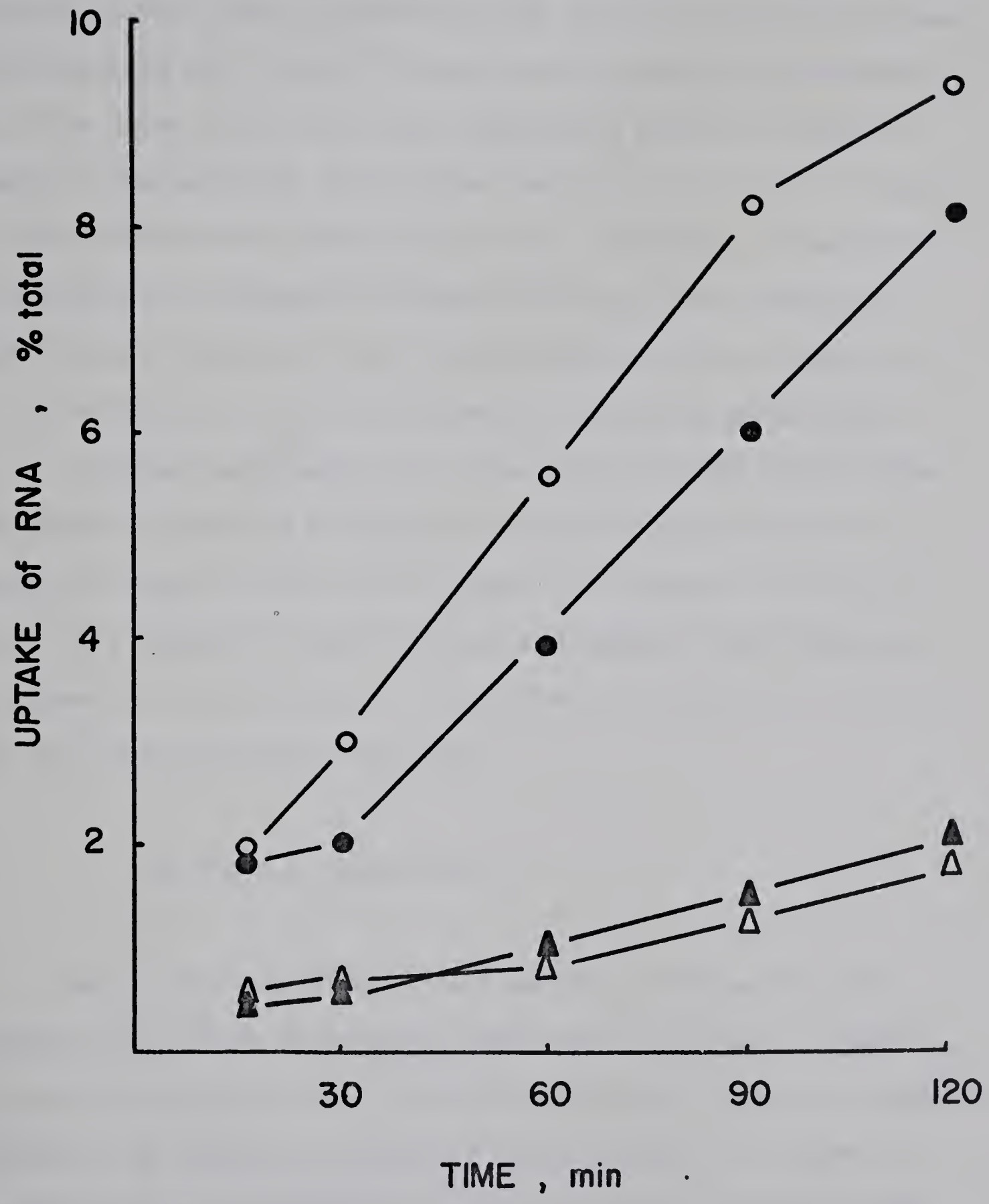

t-RNA
UMP
$A_{260\,nm}$
2
1
1
10
20
30
40
45
SLICE NUMBER
1
20
15
10
5
CPM , x $10^{-1}$

FIGURE 14

UPTAKE OF DEGRADED RNA BY EHRLICH ASCITES CELLS

Ehrlich ascites cells were incubated with 60 μg/ml degraded RNA in the presence ( ▲ ) and in the absence ( Δ ) of 5 μg/ml actinomycin D. Cells were also incubated with the same amount of undegraded RNA in the presence ( ● ) and absence ( O ) of actinomycin D.


UPTAKE of RNA , % total
10
8
6
4
2
30
60
90
120
TIME , min

the cells was measured. As a control, the cells were incubated with the same concentration of RNA which was not treated with RNase A. Incubation was carried out in the absence and presence of 5 μg/ml actinomycin D to determine to what extent inhibition of RNA synthesis affects the uptake of the degraded RNA. Figure 14 shows that the uptake of degraded RNA was in the order of 2% of the total RNA after 2 hours of incubation. Uptake of undegraded RNA, on the other hand, was in the order of 8% of the total RNA after the same period of time. Therefore, it seems that cells take up the degradation products to a much lesser extent than large molecular weight RNA. The results also show that actinomycin D had little effect on the rate of uptake of intact and degraded RNA.

A typical experiment in the determination of RNA uptake therefore involved incubating a suspension of 5% Ehrlich ascites cells in "modified" Fischer's medium with 60 μg/ml high molecular weight RNA. Samples were removed at timed intervals and washed by centrifugation and suspension with solution 5. The radioactivity associated with the cells was used to estimate RNA uptake.

## The Fate of Ingested RNA

Once it was established that a portion of the exogenous RNA incubated with Ehrlich ascites cells was taken up, it was of interest to study the fate of this RNA. The RNA might remain intact, as suggested by Galand et al (1966) and Galand and Ledoux (1966), or it might be degraded and then the degradation products could be incorporated into mammalian RNA. Since the uptake of exogenous RNA by Ehrlich ascites cells was essentially unaffected by actinomycin D (Figure 7), it could mean either that ingested RNA is stable or that it is degraded and the

degradation products are retained within the cells during washing to remove extracellular RNA.

Three methods were used to study the degradation of the RNA. The first was to measure the rate at which the radioactive RNA was degraded to acid-soluble products. The second method was to determine the electrophoretic mobility of the intracellular labelled RNA in cells exposed continuously to labelled exogenous RNA. The third method was to determine the electrophoretic mobility of intracellular labelled RNA in cells exposed to labelled exogenous RNA for only a short time and then after incubation in the absence of exogenous RNA.

It was also of interest to compare the extent of RNA degradation in cells with normal and high RNase activities to see if the elevated RNase activities were expressed.

Measurement of the Degradation of RNA to Acid-Soluble Products

Several experiments were carried out to obtain an estimate of RNA uptake, degradation of RNA and incorporation of RNA degradation products into mammalian RNA. Ehrlich ascites cells were incubated with $^{14}C$ labelled rRNA in the absence and in the presence of 5 μg/ml actinomycin D. At timed intervals, duplicate samples were removed from each incubation mixture. The cells from one set of samples were washed to remove excess RNA. In the absence of actinomycin D the radioactivity would represent intact and degraded RNA. The cells from the other samples were washed and then extracted with 4% PCA to determine the radioactivity present as acid-insoluble materiai. In the absence of actinomycin D, the radioactivity in these samples was taken to be acid-insoluble bacterial and mammalian RNA. In the presence of actinomycin D it should represent

only acid-insoluble bacterial RNA.

Two types of cells were used for these experiments: cells with normal RNase activities and cells with high RNase activities (von Tigerstrom, 1972). Both types of cells were assayed for acid and alkaline RNase II activities and for the RNase inhibitor activity. Acid RNase II activity was 10 - 16 fold higher and alkaline RNase II activity was 8 - 14 fold higher in cells with high RNase activities than in cells with normal RNase activities. Cells with normal RNase activities had approximately 40 units of inhibitor per ml of sonicate, whereas the inhibitor could not be detected in cells with high RNase activities.

Figure 15 shows the results of an experiment carried out with Ehrlich ascites cells with normal RNase activities. As shown before, actinomycin D had little effect on the amount of RNA taken up by the cells. However, the amount of radioactivity present in acid-insoluble material was substantially reduced in the presence of actinomycin D. These results can be summarized as follows:

1, RNA is taken up by the cells as indicated by curve A of Figure 15.

2, Actinomycin D has little effect on RNA uptake because curve B is similar to curve A.

3, A relatively small amount of the RNA ingested is present as acid-soluble material when mammalian RNA synthesis is not inhibited (difference between curve A and C).

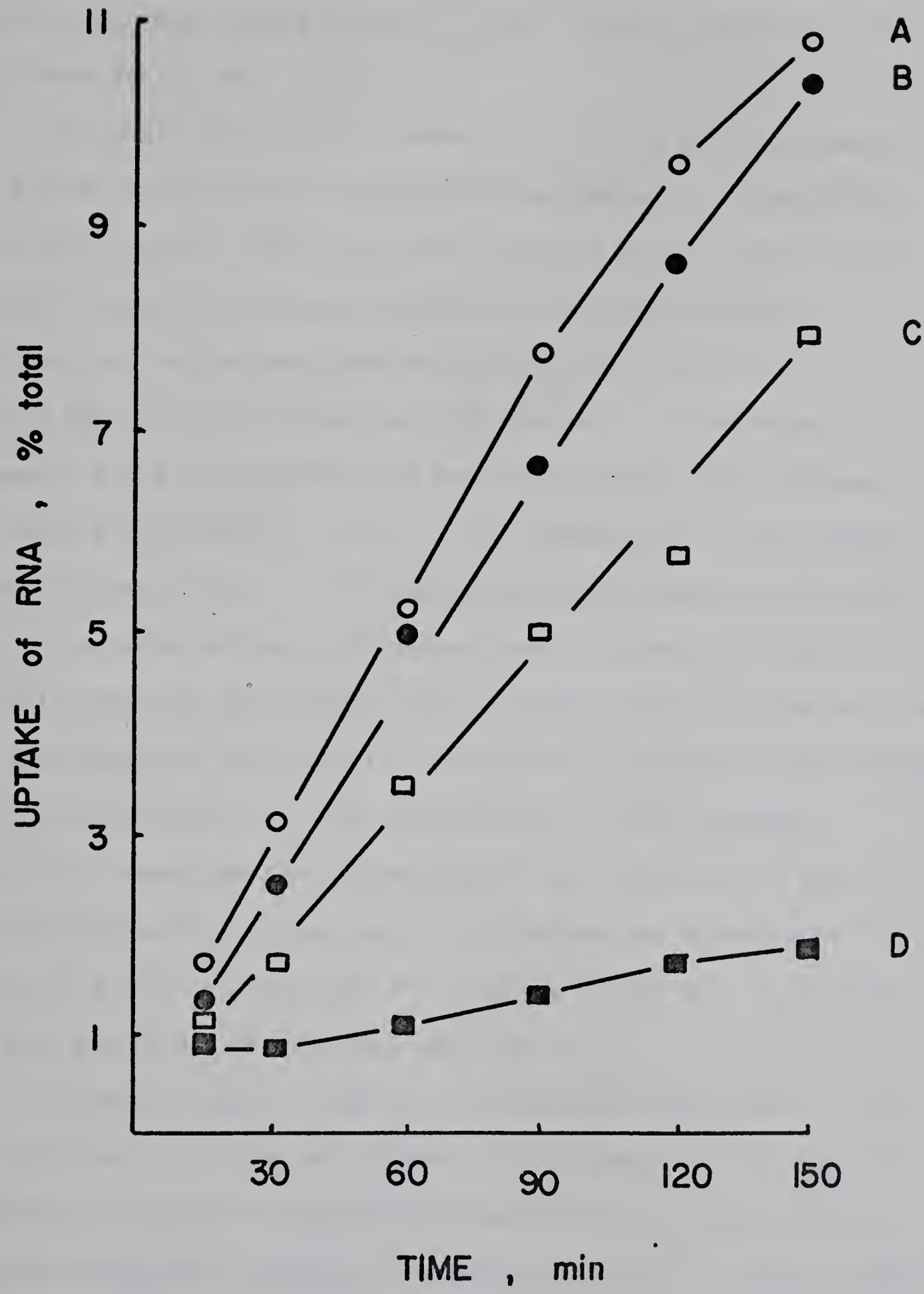
4, A large proportion of the ingested RNA is present as acid-soluble material when mammalian RNA synthesis is inhibited (difference between curves B and D).

This suggests that the bacterial RNA is taken up, degraded and the degradation products are used for the synthesis of mammalian RNA.

FIGURE 15

ESTIMATION OF RNA UPTAKE AND ACID-INSOLUBLE RADIOACTIVITY IN EHRLICH ASCITES CELLS WITH NORMAL RNASE ACTIVITIES

Ehrlich ascites cells with normal RNase activities were incubated with 60 μg/ml RNA. At the indicated times, the cells incubated in the absence of actinomycin D were washed ( O ), or washed and acid precipitated ( □ ); and the cells incubated in the presence of actinomycin D were also washed ( ● ), or washed and acid precipitated ( ■ ). The specific activity of the RNA was 0.4 μCi/mg.


UPTAKE of RNA , % total
11
9
7
5
3
1
30
60
90
120
150
TIME , min
A
B
C
D

A similar experiment to the one just described was carried out with Ehrlich ascites cells having high RNase activities. Figure 16 shows that the results were similar to those obtained with cells with normal RNase activities.

From these experiments it seems that the two cell types were quite similar with respect to uptake and degradation of ingested RNA. It was noted, however, that the results were not highly reproducible. Therefore, several experiments similar to those shown in Figures 15 and 16 were carried out and these are summarized in Table 11. The uptake of RNA is the RNA associated with the cells in the absence of actinomycin D and is expressed as a percentage of the total RNA used (e.g. curve A in Figures 15 and 16). The degradation of the ingested RNA was calculated from the difference between the uptake of RNA and the acid-insoluble radioactivity present when actinomycin D was used to inhibit mammalian RNA synthesis (e.g. curves A and D in Figures 15 and 16). This amount of acid-soluble radioactivity is given as a percentage of the total RNA taken up. The incorporation of RNA degradation products into mammalian RNA was estimated from differences in acid - insoluble radioactivity determined in the absence and presence of actinomycin D (e.g. curves C and D in Figures 15 and 16). It is also given as a percentage of the total RNA taken up.

The results shown in Table 11 indicate that RNA uptake by cells with high RNase activities was generally low. However, both types of cells seemed to degrade ingested RNA to acid-soluble products and to incorporate degradation products into mammalian RNA to a similar extent. It is possible that the low uptake of RNA by cells with high RNase activities may be due to degradation of the extracellular RNA in the

FIGURE 16

ESTIMATION OF RNA UPTAKE AND ACID-INSOLUBLE RADIOACTIVITY IN EHRLICH ASCITES CELLS WITH HIGH RNASE ACTIVITIES

Ehrlich ascites cells with high RNase activities were incubated with 60 μg/ml labelled RNA. At the indicated times, the cells incubated in the absence of actinomycin D were washed ( ○ ), or washed and acid precipitated ( □ ); and the cells incubated in the presence of actinomycin D were also washed ( ● ), or washed and acid precipitated ( ■ ). The specific activity of the RNA was 0.4 μCi/mg.

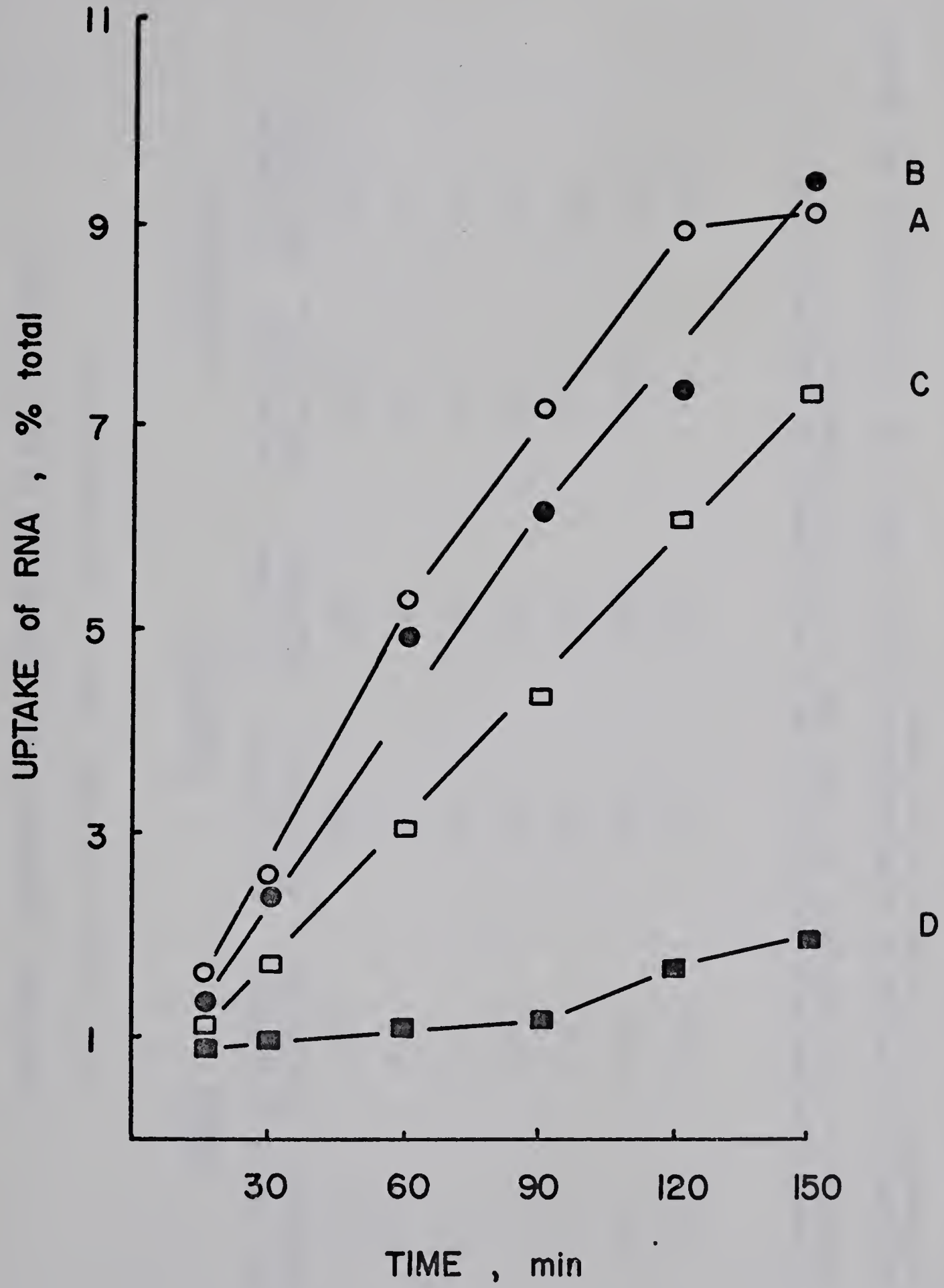

UPTAKE of RNA , % total
11
9
7
5
3
1
30
60
90
120
150
TIME , min
A
B
C
D

TABLE 11

THE UPTAKE, DEGRADATION AND INCORPORATION OF RNA BY EHRLICH ASCITES CELLS WITH NORMAL AND HIGH RNASE ACTIVITIES

| | UPTAKE | | DEGRADATION | | INCORPORATION | |
|---|---|---|---|---|---|---|
| | Normal RNase Activities | High RNase Activities | Normal RNase Activities | High RNase Activities | Normal RNase Activities | High RNase Activities |
| | 4.2% | 1.5% | 74% | 82% | 55% | 33% |
| | 4.7 | 1.5 | 75 | 87 | 47 | 10 |
| | 6.8 | 6.5 | 72 | 79 | 40 | 48 |
| | 7.6 | 4.5 | 78 | 67 | 39 | 22 |
| | 7.8 | 7.7 | 76 | 81 | 56 | 50 |
| Mean | 6.22 | 4.34 | 75.0 | 79.2 | 47.4 | 32.6 |
| Standard Deviation | 1.48 | 2.53 | 2.0 | 6.6 | 7.2 | 15.2 |

Uptake is the RNA associated with the cells in the absence of actinomycin D and is expressed as a percentage of the total RNA used in the incubation.

Degradation is defined as a percentage of the RNA taken up by the cells which has become acid-soluble in the presence of actinomycin D.

Incorporation is defined as a percentage of the acid soluble RNA which has become acid-insoluble in the absence of actinomycin D.

medium. The degraded RNA would be incorporated at a lower rate as shown in Figure 8.

## Characterization of Intracellular Labelled RNA in Cells Exposed Continuously to Labelled Exogenous RNA

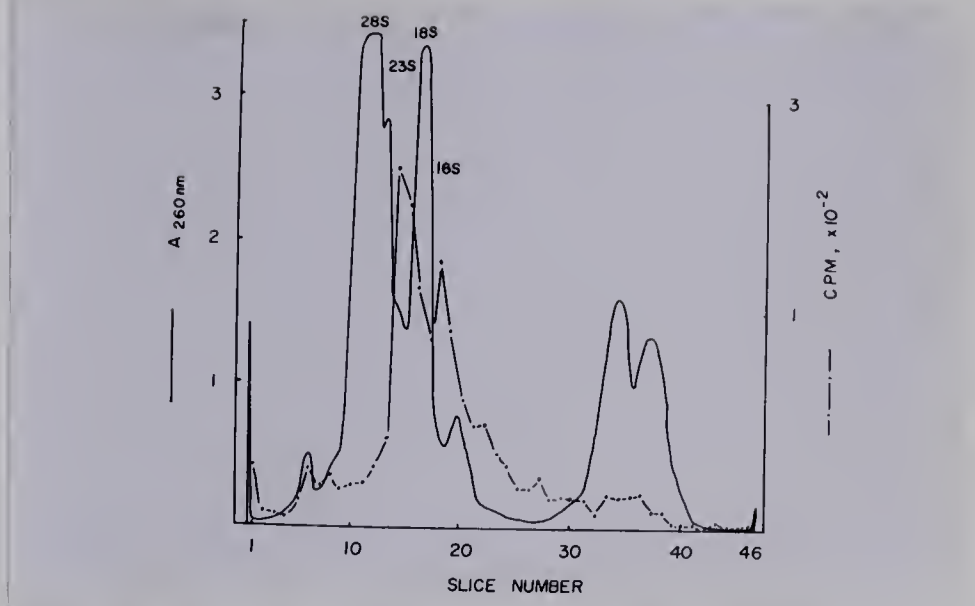
The experiments just described indicated that ingested RNA is degraded extensively in Ehrlich ascites cells with normal and high RNase activities. To confirm this and to show that the degradation products are used for mammalian RNA synthesis, it was of interest to characterize the ingested RNA during an uptake experiment. Ehrlich ascites cells with high and normal RNase activities were incubated with high specific activity RNA in the presence and absence of 5 μg/ml actinomycin D. The RNA uptake was measured as before and cells from duplicate samples were washed and used for extraction of RNA by phenol as outlined in Methods. The RNA was separated by polyacrylamide gel electrophoresis. Extensively degraded RNA is not retained by the extraction procedure and would not be detected in the profiles. The amount of ingested RNA is not great enough to be detected spectrophotometrically but the radioactive profiles should show peaks of intact bacterial RNA (23S and 16S) and partially degraded RNA in the presence of actinomycin D. In the absence of actinomycin D, if degradation products are used for mammalian RNA synthesis, incorporation of RNA should also be shown by radioactivity appearing first in the regions of 45S and 32S mammalian rRNA precursors. A comparison of these profiles should give an estimation of the degradation and incorporation of RNA.

Figures 17 and 18 show profiles of RNA extracted from Ehrlich

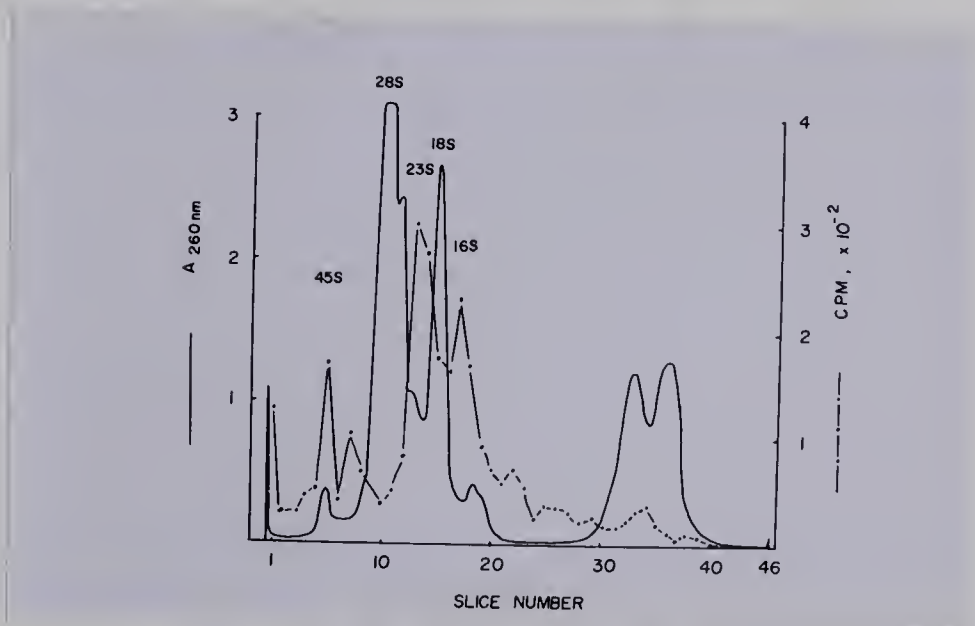
FIGURE 17

POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA EXTRACTED FROM EHRLICH ASCITES CELLS WITH NORMAL RNASE ACTIVITIES INCUBATED CONTINUOUSLY WITH RNA

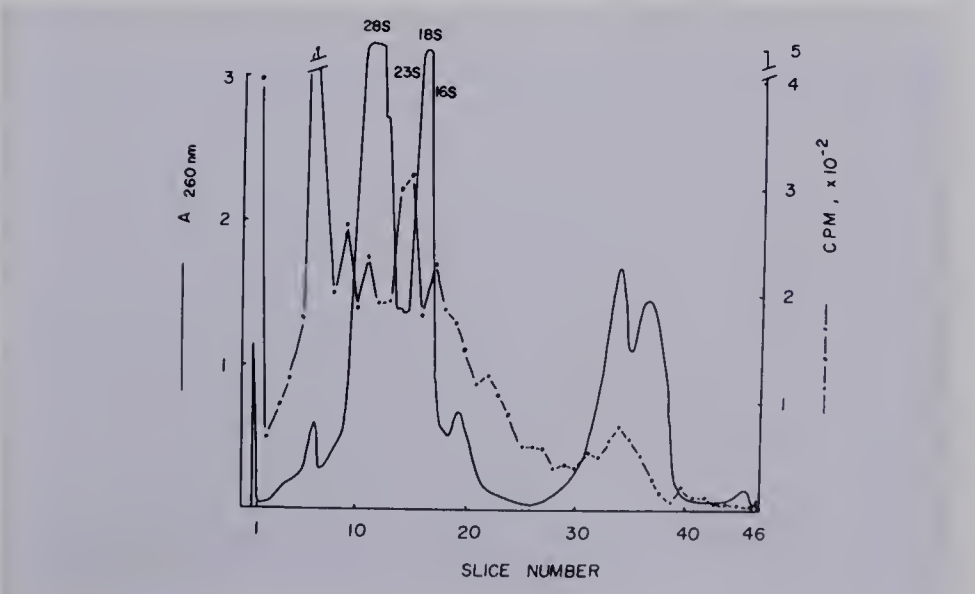
RNA extracted from 2.5 ml of Ehrlich ascites cells with normal RNase activities incubated continuously with labelled RNA for 15 minutes (Figure 17a), 30 minutes (Figure 17b), and 90 minutes (Figure 17c) was separated by polyacrylamide gel electrophoresis. The specific activity of the RNA was 15 μCi/mg.


28S
18S
23S
16S
A 260nm
CPM, x10⁻²
SLICE NUMBER

a


28S
18S
23S
16S
45S
A 260nm
CPM, x10⁻²
SLICE NUMBER

b

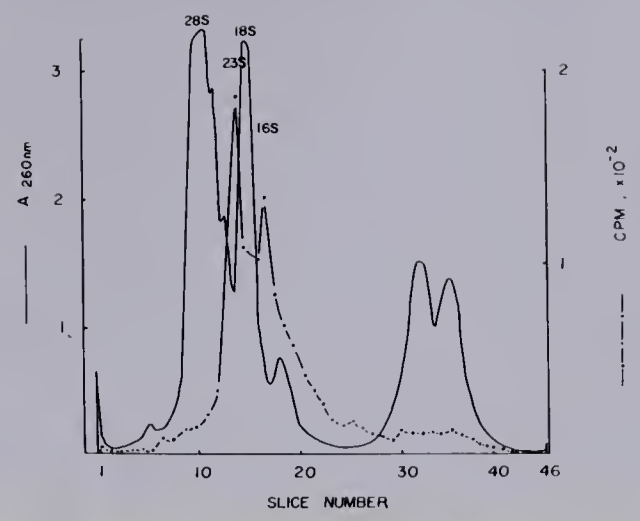

28S
18S
23S
16S
A 260nm
CPM, x10⁻²
SLICE NUMBER

c

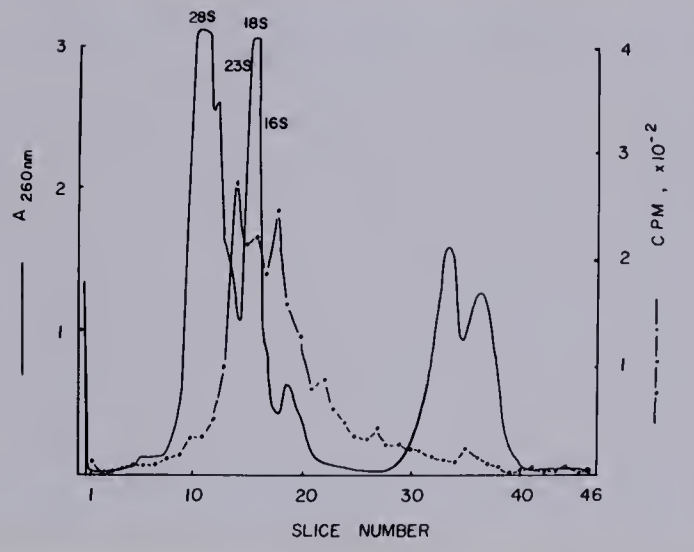
FIGURE 18

POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA EXTRACTED FROM EHRLICH ASCITES CELLS WITH NORMAL RNASE ACTIVITIES INCUBATED CONTINUOUSLY WITH RNA IN THE PRESENCE OF ACTINOMYCIN D

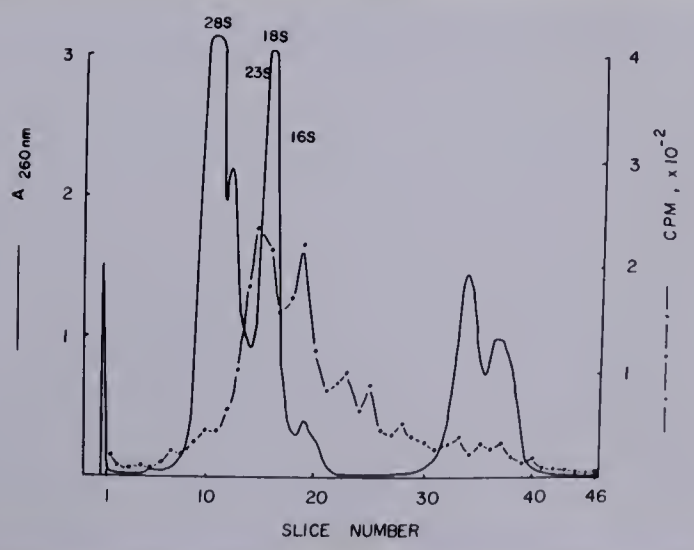
RNA extracted from 2.5 ml of Ehrlich ascites cells with normal RNase activities incubated continuously with labelled RNA in the presence of actinomycin D for 15 minutes (Figure 18a), 30 minutes (Figure 18b), and 90 minutes (Figure 18c) was separated by polyacrylamide gel electrophoresis. The specific activity of the RNA was 15 μCi/mg.


28S
18S
23S
16S
A 260nm
CPM , x10⁻²
SLICE NUMBER

a


28S
18S
23S
16S
A 260nm
CPM , x10⁻²
SLICE NUMBER

b


28S
18S
23S
16S
A 260nm
CPM , x10⁻²
SLICE NUMBER

c

ascites cells with normal RNase activities at various times during incubation in the absence and presence of actinomycin D respectively. These profiles show that more RNA was taken up as incubation progressed and the intact 23S and 16S bacterial rRNA peaks can be distinguished from the mammalian 28S and 18S rRNA by their different mobilities. In the absence of actinomycin D, incorporation of RNA into mammalian RNA was indicated by radioactivity appearing in the ribosomal RNA precursor regions 30 minutes and 90 minutes after exposure to RNA (Figures 17b and 17c). In the presence of actinomycin D the results show that the RNA becomes progressively more degraded.

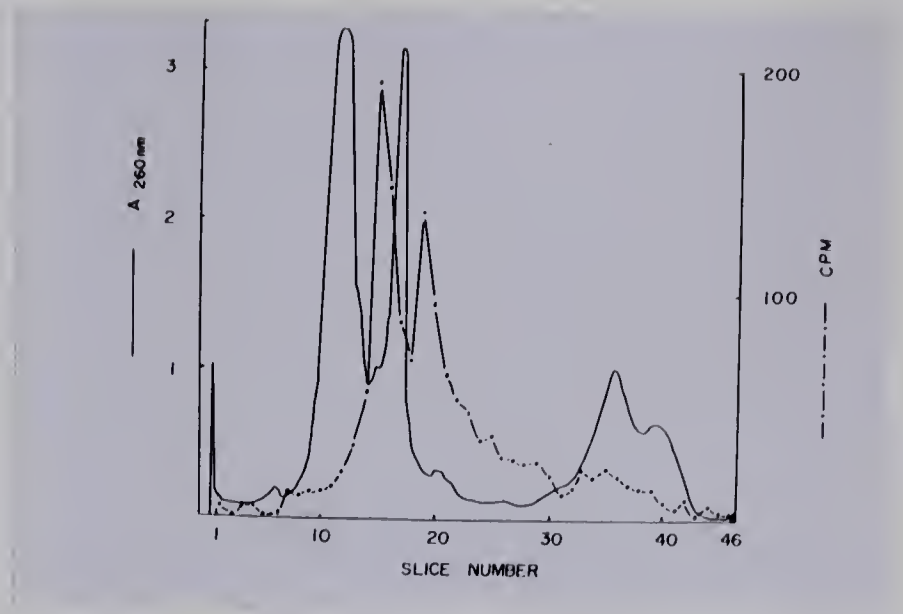
In a similar experiment cells with normal and high RNase activities were incubated continuously with labelled RNA in the presence of actinomycin D. Figures 19 and 20 show profiles of RNA extracted from Ehrlich ascites cells with normal and high RNase activities, respectively, after 15 minutes and 30 minutes incubation with RNA. The results indicate that both types of cells ingested a similar amount of intact bacterial rRNA. The RNA extracted from cells with high RNase activities, however, seemed to contain a larger amount of partially degraded RNA (Figure 20). This could be detected after 15 minutes and was very pronounced after 30 minutes of incubation. The increased amount of partially degraded RNA in these profiles suggested that the ingested RNA could be degraded faster in cells with high RNase activities than in cells with normal RNase activities. The other possibility was that degradation of RNA was similar in both types of cells but that the RNA was degraded more extensively in the medium prior to its uptake when cells with high RNase activities were used.

In order to determine to what extent extracellular degradation was taking place, the RNA in the medium was analyzed by polyacrylamide

FIGURE 19

POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA EXTRACTED FROM EHRLICH ASCITES CELLS WITH NORMAL RNASE ACTIVITIES INCUBATED CONTINUOUSLY WITH RNA IN THE PRESENCE OF ACTINOMYCIN D

RNA extracted from 2.5 ml of Ehrlich ascites cells with normal RNase activities incubated continuously with labelled RNA in the presence of actinomycin D for 15 minutes (Figure 19a), and 30 minutes (Figure 19b) was separated by polyacrylamide gel electrophoresis. The specific activity of the RNA was 13 μCi/mg.


A 260nm
CPM
SLICE NUMBER
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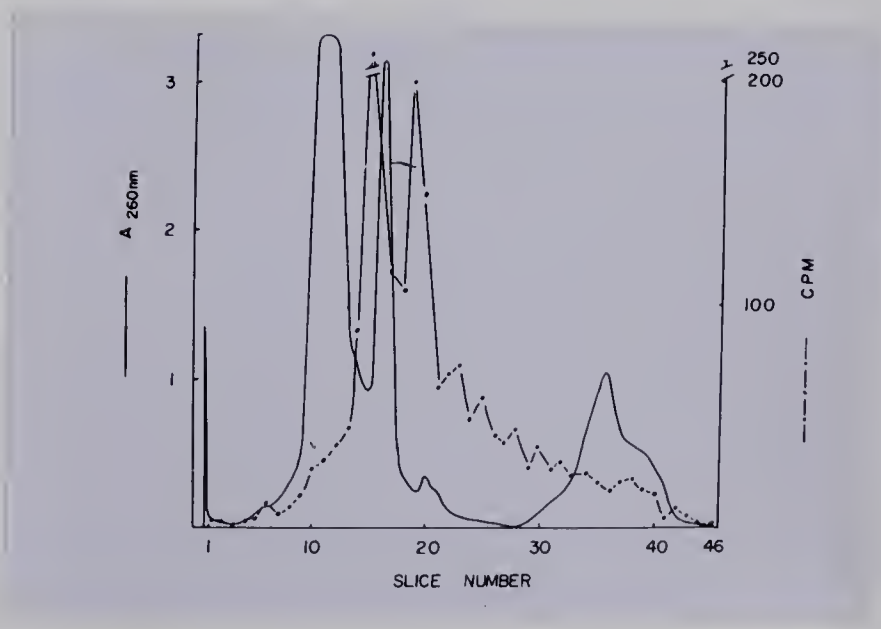
10
20
30
40
46
100
200
1
2
3

a

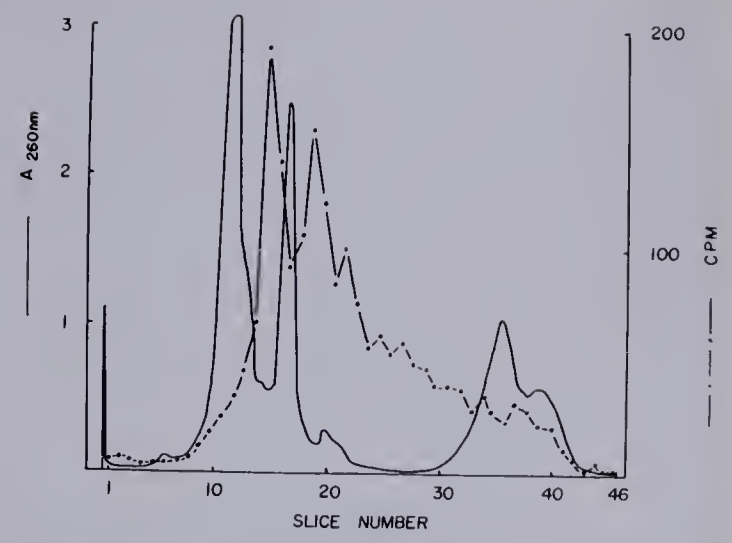

A 260nm
CPM
SLICE NUMBER
1
10
20
30
40
46
100
200
250
1
2
3

b

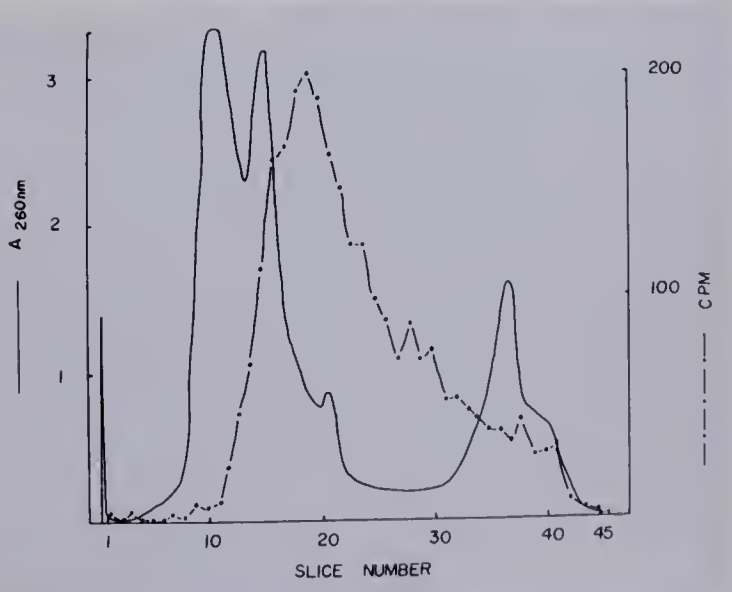
FIGURE 20

POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA EXTRACTED FROM EHRLICH ASCITES CELLS WITH HIGH RNASE ACTIVITIES INCUBATED CONTINUOUSLY WITH RNA IN THE PRESENCE OF ACTINOMYCIN D

RNA extracted from 2.5 ml of Ehrlich ascites cells with high RNase activities incubated continuously with labelled RNA in the presence of actinomycin D for 15 minutes (Figure 20a), and 30 minutes (Figure 20b) was separated by polyacrylamide gel electrophoresis. The specific activity of the RNA was 13 μCi/mg.


A 260nm
CPM
SLICE NUMBER
1
10
20
30
40
46
100
200
1
2
3

a


A 260nm
CPM
SLICE NUMBER
1
10
20
30
40
45
100
200
1
2
3

b

gel electrophoresis and the medium was assayed for RNase activities. It was found that during the incubation of cells with normal RNase activities, the alkaline RNase II activity in the medium increased 4 fold in an hour of incubation (from 0.0005 units to 0.0022 units per ml of medium). The effect of this alkaline RNase, and other RNases likely to be in the medium, on the small amount of extracellular RNA was significant since the amount of RNA smaller than 16S rRNA was found to increase from 15% to 21% and to 26% after 10, 30 and 60 minutes of incubation. The degradation of extracellular RNA was even more extensive during the incubation of cells with high RNase activities as shown by profiles of RNA in the medium after 15 minutes and 30 minutes of incubation (Figure 21). The partially degraded RNA is likely taken up by the cells (Figure 12) along with intact RNA, and it would also appear on profiles of RNA extracted from cells and could be easily mistaken as the result of intracellular RNA degradation.

Although the rate of extracellular degradation seemed much higher with cells having high RNase activities it was not clear whether the rate of degradation of ingested RNA in these cells was significantly faster than degradation in cells with normal RNase activities.

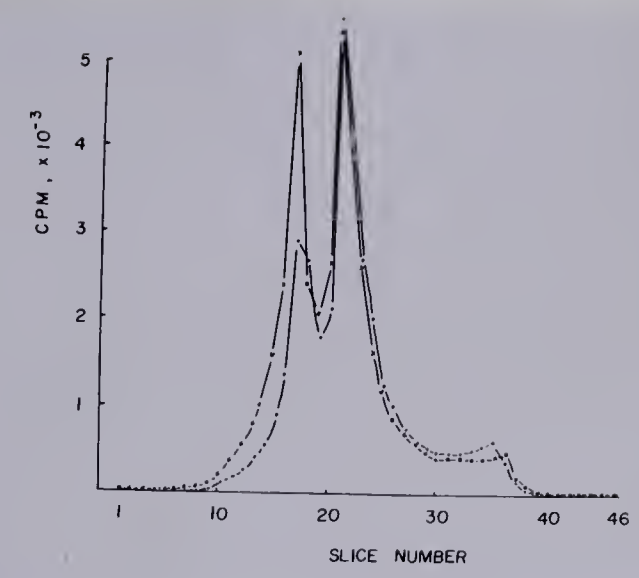
## Characterization of Intracellular Labelled RNA in Cells Exposed to Labelled Exogenous RNA for a Brief Period

In order to determine the extent of intracellular degradation of ingested RNA more accurately, steps were taken to minimize the effect of RNases in the medium. The cells were washed 6 times rather than the usual 3 - 4 times during harvesting to remove excess RNase. Furthermore, since little degradation of extracellular RNA occurred during the initial

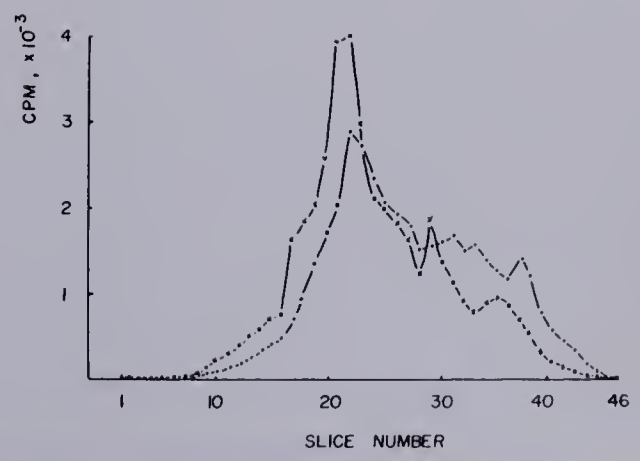
FIGURE 21

POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA
ISOLATED FROM THE INCUBATION MEDIUM

Ehrlich ascites cells with normal RNase activities (Figure 21a) and cells with high RNase activities (Figure 21b) were incubated for 15 (×—×) and 30 minutes (•—•). The cells were removed by centrifugation and 100 µl of the supernatant containing about 6 µg of RNA was separated by polyacrylamide gel electrophoresis. About 2 mm gel slices were cut and the radioactivity in the slices was determined.


CPM, x 10⁻³
5
4
3
2
1
1
10
20
30
40
46
SLICE NUMBER

a


CPM, x 10⁻³
4
3
2
1
1
10
20
30
40
46
SLICE NUMBER

b

15 - 20 minutes of incubation, the cells were exposed to RNA only for short periods. After incubation with labelled RNA for 20 minutes, the cells were centrifuged at 1,000 x g, resuspended in fresh medium at 0° to 4°, washed once, and then incubated at 37° without RNA in the medium. The fate of the relatively small amount of RNA ingested by the cells was traced by polyacrylamide gel electrophoresis. Duplicate cell samples were taken before centrifugation, immediately after resuspension in fresh medium without RNA, and 60 minutes after incubation in the fresh medium without RNA. One set of samples was used to measure RNA uptake and RNA was extracted from the other cell samples and characterized by polyacrylamide gel electrophoresis. As a control, the procedure was carried out in the same way but the cells were resuspended and washed in the original medium containing labelled RNA.

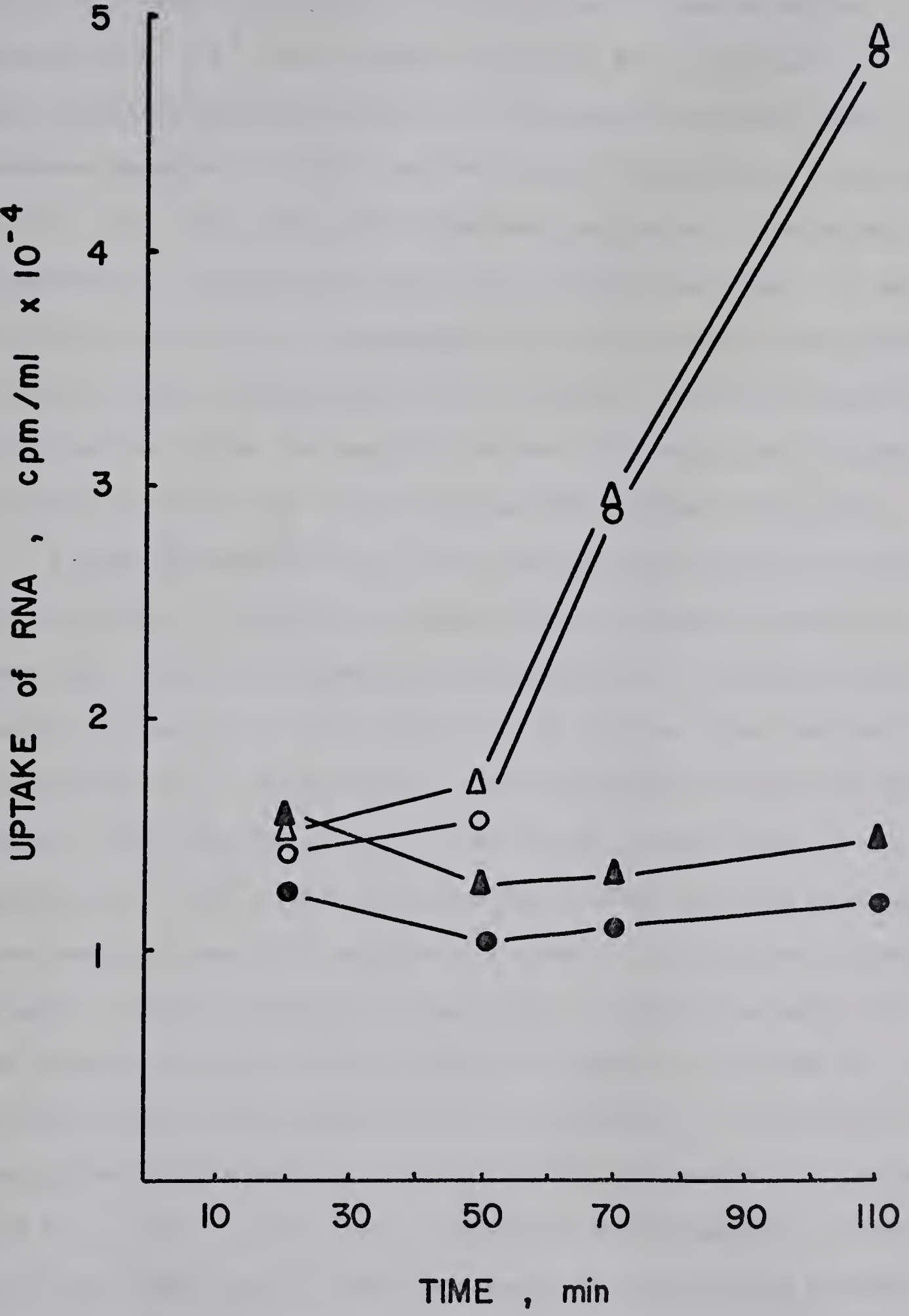
The labelled RNA present in the medium after 20 minutes incubation was also analyzed by polyacrylamide gel electrophoresis. A comparison of the profiles of RNA in the medium and the RNA extracted from the cells incubated for 20 minutes with labelled RNA should give an estimate of the incorporation and degradation of relatively intact bacterial rRNA. The degradation of the labelled RNA during subsequent incubation in the absence of exogenous RNA could be followed by electrophoresis of extracted RNA and compared to the profiles obtained when cells were incubated continuously with labelled RNA.

The experiment was conducted with Ehrlich ascites cells having normal and high RNase activities and in the presence of 5 μg/ml actinomycin D. Figure 22 shows the radioactivity associated with cells after 20 minutes of incubation, after washing and resuspension, and after continued incubation in the presence or absence of exogenous RNA.

FIGURE 22

UPTAKE OF RNA BY EHRLICH ASCITES CELLS WITH NORMAL AND HIGH RNASE ACTIVITIES IN CONTINUOUS PRESENCE OF LABELLED RNA AND AFTER BRIEF INCUBATION WITH LABELLED RNA

Ehrlich ascites cells with normal RNase activities were incubated with labelled RNA for 20 minutes in the presence of actinomycin D. The cells were centrifuged and washed and suspended in fresh medium without exogenous RNA for re-incubation ( ● ). Washing and resuspension of the cells were carried out from 20 minutes to 50 minutes. The same procedure was followed using cells with high RNase activities ( ▲ ). Ehrlich ascites cells with normal ( O ) and high ( Δ ) RNase activities were also incubated for 20 minutes with labelled RNA in the presence of actinomycin D, centrifuged, washed and resuspended in the original medium containing exogenous RNA.


UPTAKE of RNA , cpm/ml x $10^{-4}$
5
4
3
2
1
10
30
50
70
90
110
TIME , min

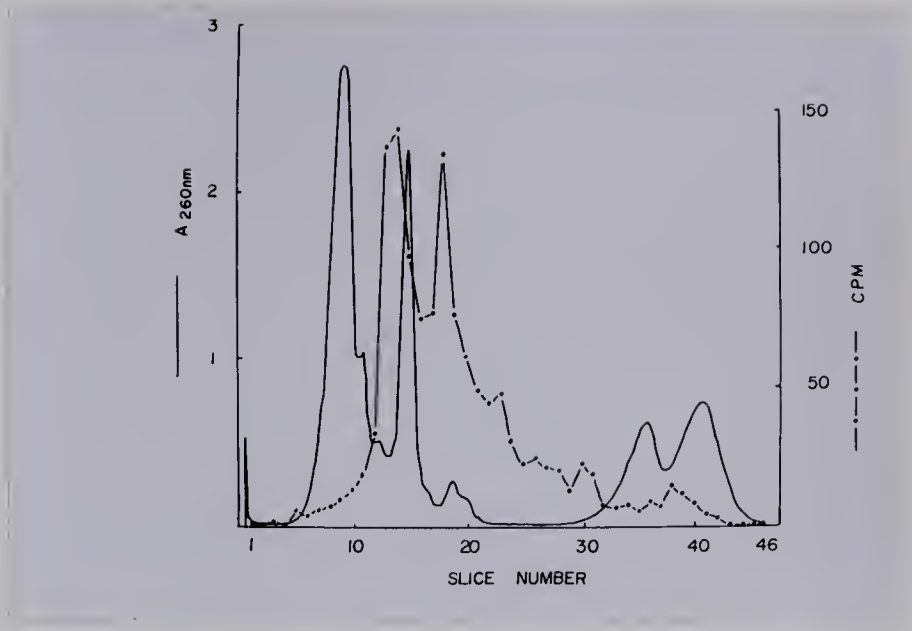
When cells were washed and resuspended with exogenous RNA, the RNA was taken up at a very much reduced rate during centrifugation and resuspension at 0° - 4°. On continued incubation at 37°, the cells resumed ingesting RNA at a high rate. This suggests that RNA uptake is temperature dependent although some RNA can still be taken up or adsorbed at 0°- 4°. When cells were centrifuged and resuspended in the absence of exogenous RNA, the RNA associated with the cells decreased. It was likely that the RNA which had adsorbed on the cell membrane dissociated from the cells upon resuspension in fresh medium. Continued incubation in fresh medium did not decrease the radioactivity associated with the cells suggesting that most of the ingested RNA remained in the cells.

Figure 23 shows profiles of RNA extracted from cells with normal RNase activities. The RNA was extracted after 20 minutes incubation (Figure 23a), after centrifugation and resuspension in the same medium containing exogenous RNA (Figure 23b), and 60 minutes after resuspension and incubation at 37° (Figure 23c). The incorporation of RNA continued throughout the incubation periods. A portion of the RNA taken up was intact bacterial rRNA especially during the initial 20 minute incubation and the amount of partially degraded RNA seemed to increase as incubation progressed. Figure 24 shows profiles of RNA extracted from cells with normal RNase activities after 20 minutes of incubation with RNA (Fig. 24a), after centrifugation and resuspension in fresh medium in the absence of exogenous RNA (Figure 24b),and 60 minutes after resuspension and incubation at 37° (Figure 24c). After the centrifugation and resuspension of the cells (Figure 24b) much less RNA was present in the cells and the RNA isolated after 60 minutes incubation in the absence of exogenous RNA was substantially reduced. Therefore, degradation of ingested RNA

FIGURE 23

POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA EXTRACTED FROM EHRLICH ASCITES CELLS WITH NORMAL RNASE ACTIVITIES IN THE CONTINUOUS PRESENCE OF RNA

Ehrlich ascites cells with normal RNase activities were incubated with labelled RNA in the presence of actinomycin D for 20 minutes (Figure 23a), centrifuged and resuspended at 0° - 4° in the same medium containing labelled RNA (Figure 23b), and incubated for a further 60 minutes at 37° (Figure 23c). RNA extracted from 2.5 ml of these cells was separated by polyacrylamide gel electrophoresis. The specific activity of the exogenous RNA was 13 μCi/mg.


A 260nm
CPM
SLICE NUMBER
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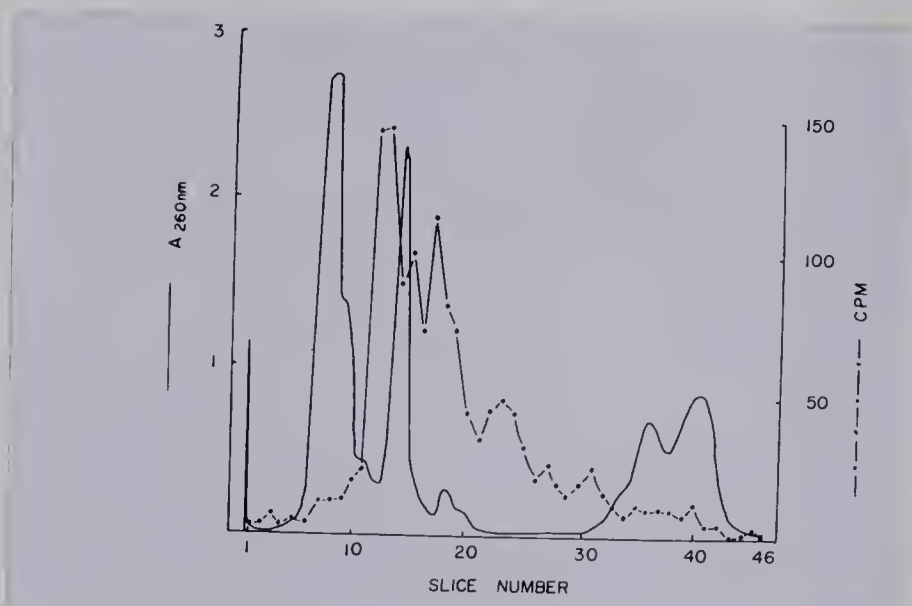
10
20
30
40
46
1
2
3
50
100
150

a


A 260nm
CPM
SLICE NUMBER
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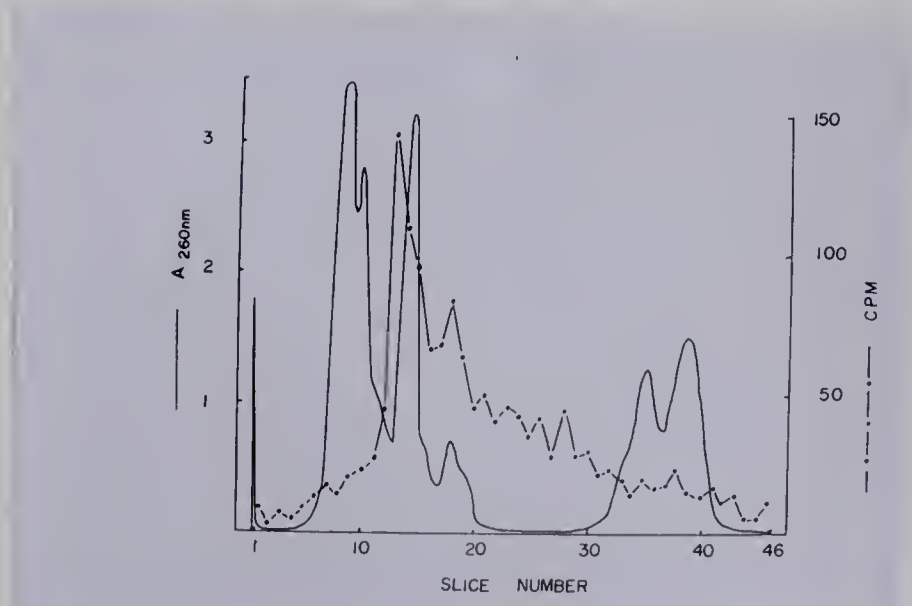
10
20
30
40
46
1
2
3
50
100
150

b

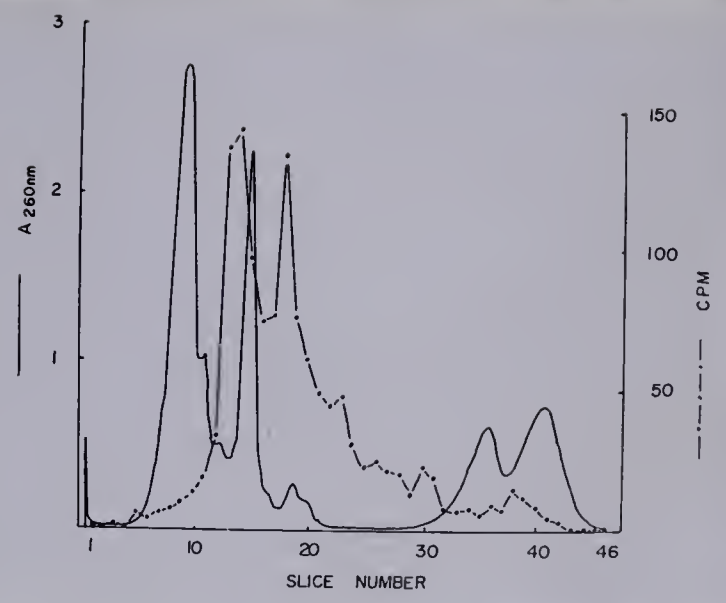

A 260nm
CPM
SLICE NUMBER
1
10
20
30
40
46
1
2
3
50
100
150

c

FIGURE 24

POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA EXTRACTED FROM EHRLICH ASCITES CELLS WITH NORMAL RNASE ACTIVITIES AFTER BRIEF EXPOSURE TO RNA

Ehrlich ascites cells with normal RNase activities were incubated with labelled RNA in the presence of actinomycin D for 20 minutes (Figure 24a), centrifuged and resuspended in fresh medium (Figure 24b), and incubated for a further 60 minutes at 37° (Figure 24c). RNA extracted from 2.5 ml of these cells was separated by polyacrylamide gel electrophoresis. The specific activity of the exogenous RNA was 13 μCi/mg.


A 260nm
CPM
SLICE NUMBER
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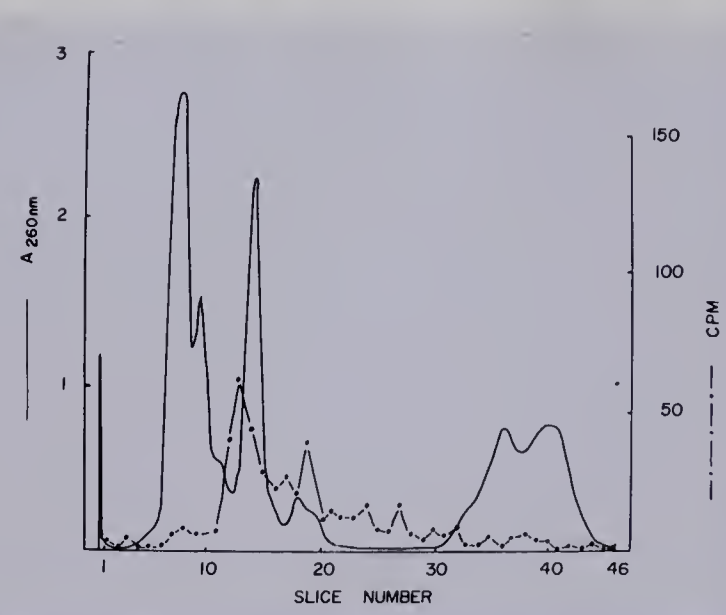
10
20
30
40
46
50
100
150
1
2
3

a


A 260nm
CPM
SLICE NUMBER
1
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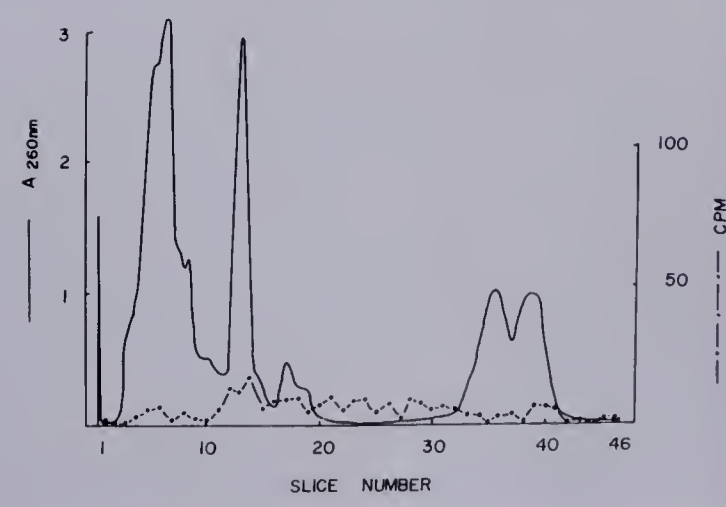
20
30
40
46
50
100
150
1
2
3

b


A 260nm
CPM
SLICE NUMBER
1
10
20
30
40
46
50
100
1
2
3

c

occurred very rapidly even in cells with normal RNase activities.

The results of an experiment which was identical except that Ehrlich ascites cells with high RNase activities were used, are shown in Figures 25 and 26. When cells were incubated with labelled RNA for 20 minutes, centrifuged and resuspended in the same medium containing labelled RNA, the uptake and the profiles of the RNA were similar to those for normal cells (Figures 23a, b and c) except that the profiles contained less intact and more partially degraded RNA. Since the RNA in the medium with cells having high RNase activities is degraded more extensively than the RNA in the medium with cells having normal RNase activities (Figure 27), it is likely that these profiles represent uptake of partially degraded as well as intact RNA. The profiles of the RNA extracted from the cells with high RNase activities (Figure 26) show that the degradation of ingested RNA occurs rapidly and was similar to the degradation in cells with normal RNase activities.

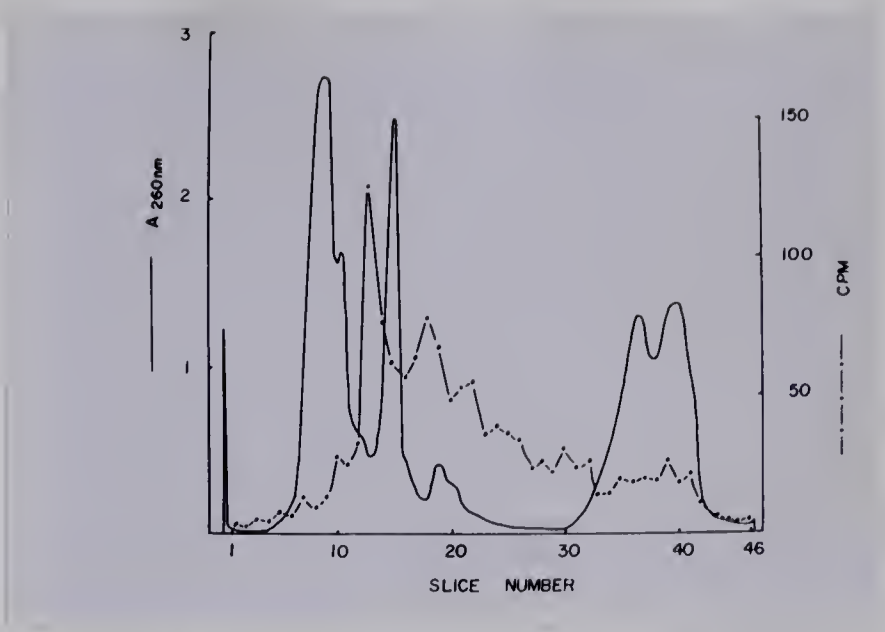
A similar experiment as the one described above was carried out with both cells types in the absence of actinomycin D. The results obtained from this experiment also showed that RNA degradation in the medium was faster for cells with high RNase activities as compared to cells with normal RNase activities. No significant differences could be observed with respect to degradation of ingested RNA. However, the regions corresponding to mammalian rRNA precursors (45S and 32S) were labelled at various times of incubation suggesting that degradation products were incorporated into mammalian RNA, confirming earlier results (Figures 15, 16 and 17).

The investigation of the fate of ingested RNA showed that the RNA was degraded rapidly in cells with normal and in cells with high

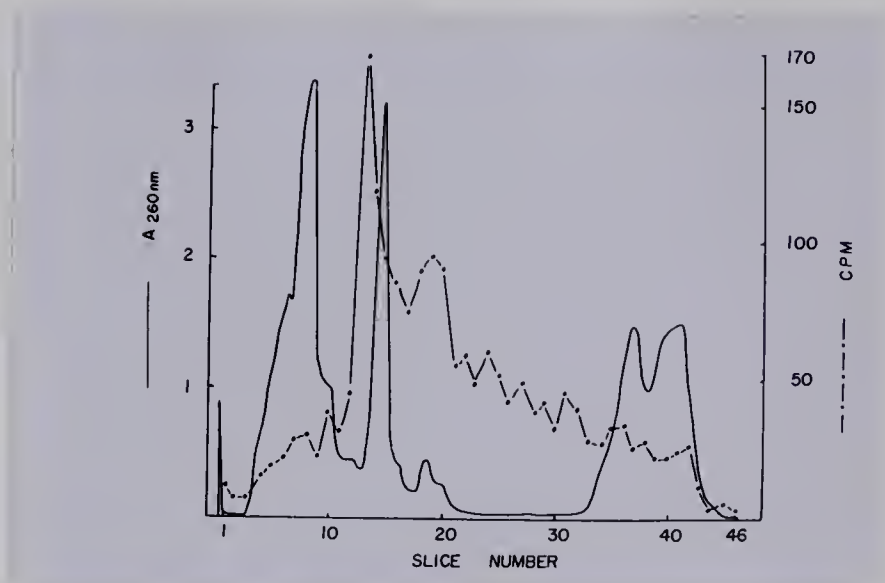
FIGURE 25

POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA EXTRACTED FROM EHRLICH ASCITES CELLS WITH HIGH RNASE ACTIVITIES IN THE CONTINUOUS PRESENCE OF RNA

Ehrlich ascites cells with high RNase activities were incubated with labelled RNA in the presence of actinomycin D for 20 minutes (Figure 25a), centrifuged and resuspended in the same medium containing labelled RNA (Figure 25b), incubated for a further 60 minutes at 37° (Figure 25c). RNA extracted from 2.5 ml of these cells was separated by polyacrylamide gel electrophoresis. The specific activity of the exogenous RNA was 13 μCi/mg.


A 260nm
CPM
SLICE NUMBER
1
10
20
30
40
46
1
2
3
50
100
150

a


A 260nm
CPM
SLICE NUMBER
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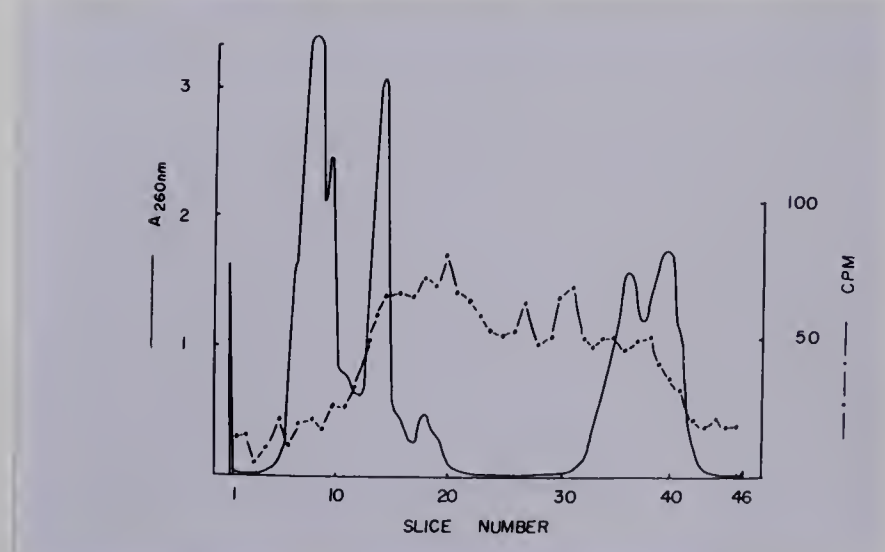
20
30
40
46
1
2
3
50
100
150
170

b

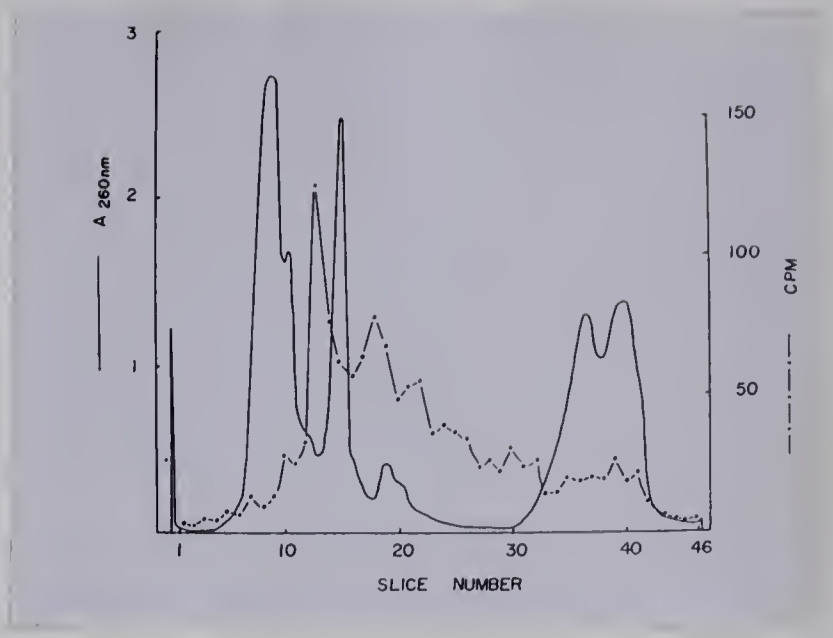

A 260nm
CPM
SLICE NUMBER
1
10
20
30
40
46
1
2
3
50
100

c

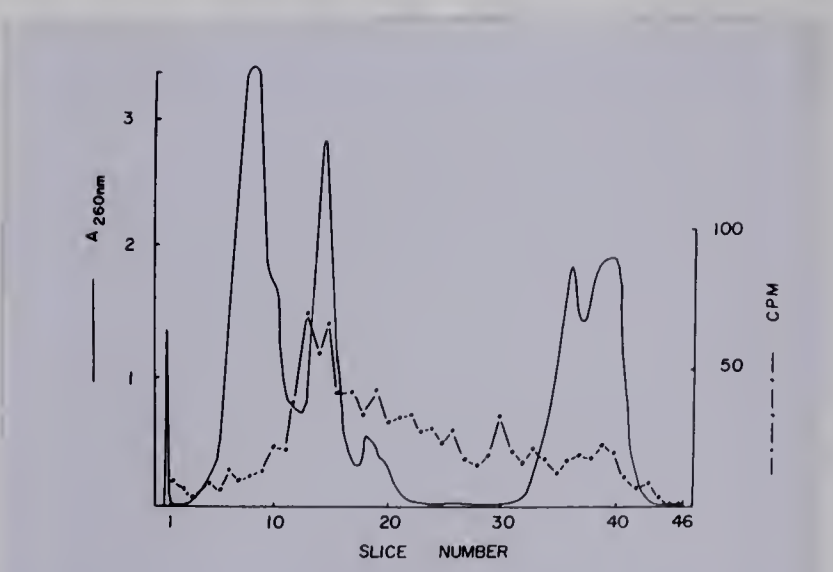
FIGURE 26

POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA EXTRACTED FROM EHRLICH ASCITES CELLS WITH HIGH RNASE ACTIVITIES AFTER BRIEF EXPOSURE TO RNA

Ehrlich ascites cells with high RNase activities were incubated with labelled RNA for 20 minutes in the presence of actinomycin D (Figure 26a), centrifuged and resuspended in fresh medium (Figure 26b), and incubated for a further 60 minutes at 37° (Figure 26c). RNA extracted from 2.5 ml of these cells was separated by polyacrylamide gel electrophoresis. The specific activity of the exogenous RNA was 13 μCi/mg.


A 260nm
CPM
SLICE NUMBER
1
10
20
30
40
46
1
2
3
50
100
150

a


A 260nm
CPM
SLICE NUMBER
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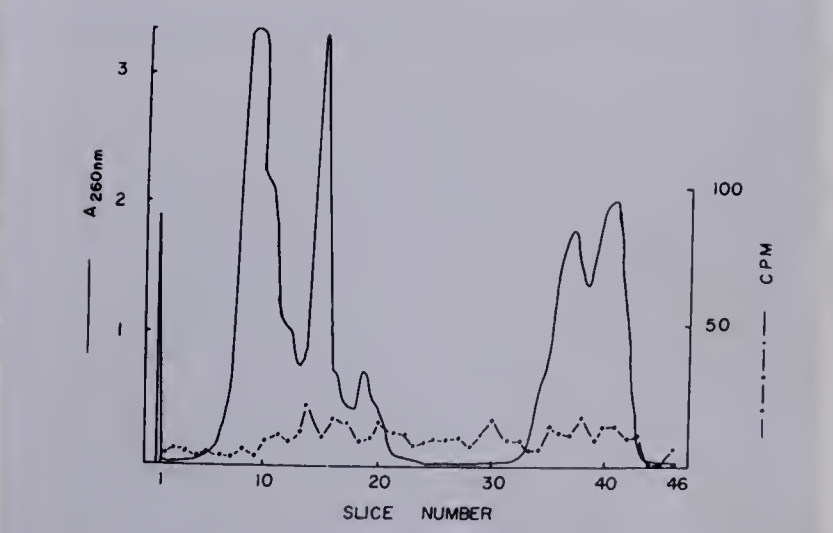
30
40
46
1
2
3
50
100

b

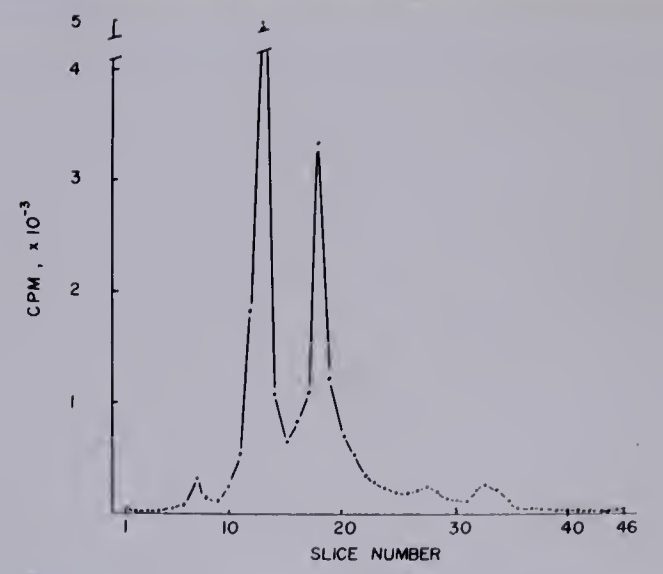

A 260nm
CPM
SLICE NUMBER
1
10
20
30
40
46
1
2
3
50
100

c

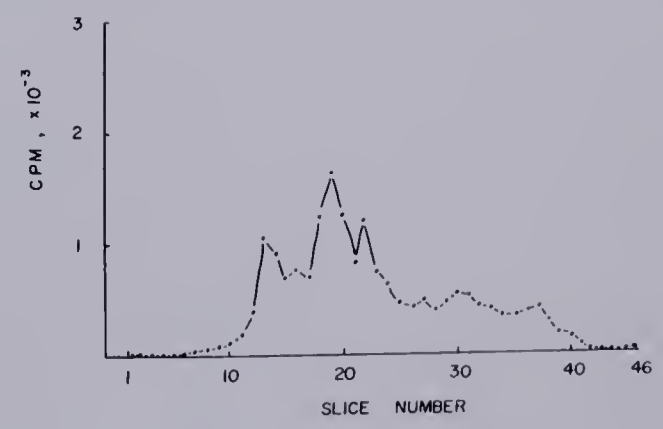
FIGURE 27

CHARACTERISTICS OF RNA ISOLATED FROM THE MEDIUM

After 20 minutes incubation of Ehrlich ascites cells with normal (Figure 27a) and high RNase activities (Figure 27b), the cells were centrifuged and 50 µl of the supernatant containing 3 µg RNA was separated by polyacrylamide gel electrophoresis. Approximately 2 mm gel slices were cut and the radioactivity of the slices was counted.


CPM , x 10⁻³
5
4
3
2
1
1
10
20
30
40
46
SLICE NUMBER

a


CPM , x 10⁻³
3
2
1
1
10
20
30
40
46
SLICE NUMBER

b

RNase activities. The degradation products were utilized for the synthesis of Ehrlich ascites cell RNA in the absence of actinomycin D. In the presence of actinomycin D acid-soluble degradation products or their metabolites accumulated within the cells. The results also showed that the exogenous RNA is degraded extracellularly at a significant rate, especially when cells with high RNase activities were used.

# DISCUSSION

The object of this study was to investigate the uptake of RNA and the extent of degradation of ingested RNA by Ehrlich ascites cells with normal and with high RNase activities. The first part of this work was primarily concerned with measuring RNA uptake and with establishing optimum conditions for ingestion of exogenous RNA by Ehrlich ascites cells. The second part was concerned with the intracellular degradation of the ingested RNA.

One of the major criticisms directed against earlier works on RNA uptake by mammalian cells was the failure to characterize the RNA employed in the experiments (Niu et al, 1961; Yoon, 1965). In this project, high molecular weight bacterial RNA separated by Bio-Gel P-60 chromatography was used. It contained 85 - 92% rRNA and the remaining components were also mainly of high molecular weight. The purified bacterial rRNA could be prepared satisfactorily with a high specific activity and it could be distinguished from mammalian RNA by electrophoresis. This facilitated later studies on the fate of the RNA ingested by Ehrlich ascites cells.

In order to estimate the RNA uptake by Ehrlich ascites cells, the RNA associated non-specifically with the cells must be removed. Washing the cells on filter discs seemed unable to disiodge much of the RNA adsorbed on the cell mambrane. Washing the cells repeatedly by centrifugation and resuspension, however, seemed to yield consistent and reproducible results. The vigorous washing procedure apparently had no harmful effect on the cells and after washing the cells 3-4 times, no additional RNA was removed from the cells. The radioactivity associated with the washed cells was measured and the term "uptake of RNA" denotes the total RNA associated with the washed cells.

It is likely that some RNA adsorbed on the cell membrane would be included in the estimation of RNA uptake by Ehrlich ascites cells. No attempts, however, were made to estimate the absolute amount of RNA taken up by the cells as opposed to the amount adsorbed on cell membrane because it seemed that the amount of RNA adsorbed was too small to cause concern. Indirect evidence suggested that a large portion of the RNA associated with the cells was taken up intracellularly. First, after an initial rapid uptake of RNA (0 - 10 seconds), uptake was slow and approximately linear with time (Figure 6). When 60 μg/ml rRNA was used, only 0.5% of the total RNA was taken up during the initial period as compared to 7 - 9% after 2 hours of incubation. The initial rapid uptake was probably due to adsorption and the slower uptake of RNA due to intracellular ingestion. Secondly, when the RNA concentration in the medium was increased, the radioactivity associated with the cells during the initial period increased but proportionally less RNA was taken up during incubation. Thirdly, a large portion of the RNA taken up became acid soluble within a short time (Figures 15 and 16) but actinomycin D had little effect on the uptake of RNA (Figure 7). The similar rate of uptake in the presence and absence of actinomycin D suggested that RNA is taken up intracellularly, degraded and the degradation products are not removed from the cells during washing.

No attempts were made to use RNase to remove the adsorbed RNA since RNase was reported to penetrate ascites cells easily either by pinocytosis or by diffusion (Ledoux, 1956). It was thought that this might interfere with later studies on the fate of the ingested RNA although more recent reports indicated that RNase does not enter the cells. Treatment with 100 μg/ml RNase did not degrade intracellular

RNA (Bausek and Merigan, 1969), and the RNA taken up by Nelson's ascites cells was protected from the RNase (Yoon, 1965).

The mechanism by which RNA is taken up by mammalian cells is not clear. Several reports suggested that macromolecules are ingested by pinocytosis (Easty et al, 1956). Occasional clumps of cytoplasmic radioactivity had been demonstrated by autoradiography suggesting that pinocytotic vesicles containing RNA were present after the adminstration of RNA (Schwarz and Rieke, 1962) Pinocytosis is dependent on cellular metabolism and is inhibited by metabolic inhibitors such as dinitrophenol and iodoacetate (Galand et al, 1966). In this project, iodoacetate ($10^{-3}$ M) was found to inhibit RNA uptake after a lag of 30 - 60 minutes. Other workers, however, could not find the presence of vesicle formation (Herrera et al, 1970) and Holoubek et al (1966) and Shanmugan and Bhargava (1966) reported that metabolic inhibitors have no effect on the uptake of RNA. Furthermore, poly-L-lysine increases RNA uptake (Mayhew and Juliano, 1973) but it does not affect pinocytosis (Cohn and Parks, 1966). Whether RNA is transported directly into the cells or enters by pinocytosis in vesicles, binding of RNA on the cell membrane must preceed the uptake of RNA into the cells. This binding might occur in varying degrees of intensity according to the nature of the membrane. For example, the microsomal membrane was reported to react only with DNA and poly C : poly G in vitro while the nuclear envelope binds only with poly C and ribosomal RNA. These ribonucleic acid complexes are not formed with erythrocyte "ghosts" from man, dog, pig, mouse and rat (Kubinski et al, 1972).

During the course of the investigation, the incubation medium was found to contain RNase activities which degraded the rRNA in the medium. As incubation progressed, the degradation of high molecular weight RNA would

result in an increase in the number of low molecular weight RNA molecules in the incubation medium. It was necessary therefore to determine to what extent RNA smaller than rRNA was taken up and to what extent high RNA concentrations affected RNA uptake. In the study of the uptake of low molecular weight RNA and of RNA degraded by RNase, the results suggested that RNA uptake is dependent on the concentration of RNA and Ehrlich ascites cells take up low molecular weight RNA and RNA degradation products to a lesser extent than large molecular weight RNA.

In order to increase the sensitivity of the experiments on the fate of ingested RNA, efforts were made to increase RNA uptake and to increase the amount of isotope taken up. DEAE-dextran had been used successfully by Colby and Chamberlin (1969) and by Busch et al (1972) to increase uptake. In this project, when cells were pre-incubated with 100 µg/ml DEAE-dextran before the addition of RNA, the RNA associated with the cells at zero time was very much increased. Even at a lower concentration of DEAE-dextran (40 µg/ml), the adsorption was increased without increasing the rate of RNA uptake (Figure 10). Washing the cells failed to dislodge the DEAE-dextran or the adsorbed RNA from the cells. This agrees with the results obtained by Bausek and Merigan (1969) that DEAE-dextran contributes only to the adsorption of RNA on cell membrane. It is still possible that DEAE-dextran and other polyions, by reducing or reversing the net negative charge of the tumour cells and/or by protecting the RNA from RNase in the medium, facilitate the transport of RNA in some cell types.

In other attempts to increase the uptake of RNA by Ehrlich ascites cells, experiments were carried out with various amounts of

RNA and with various cell concentrations. With higher cell concentrations, the amount of RNA taken up per cell seemed to remain unchanged. With higher RNA concentrations in the incubation medium, adsorption of RNA was increased. The rate of uptake of RNA, however, was proportionally lower (Figures 8 and 12). There seems to be competition between the RNA molecules for uptake. This agrees with reports by Schell (1971), Bishop and Abramhoff (1966), and Chin and Silverman (1967). Juliano and Mayhew (1972), however, reported that uptake of RNA is proportional to RNA concentrations up to 50 μg/ml and uptake is saturable at concentrations greater than 250 μg/ml (Herrera et al, 1970).

The uptake of RNA by Ehrlich ascites cells with high RNase activities is sometimes low (Table 11). It is likely that the RNA extracellular to these cells is degraded rapidly and when the concentration of RNA is increased, the total uptake of radioactivity is reduced. This hypothesis is substantiated by analysis of RNA and RNase activities in the medium (Figure 21). Even in the incubation of cells having normal RNase activities there is sufficient extracellular RNase to affect the integrity of the RNA in the medium. The degradation of extracellular RNA by cells with high RNase activities is even more extensive. RNase might be released to the medium either by active secretion or on lysis of the cells. Addition of the inhibitor of alkaline RNase II does not seem to inhibit the RNase in the medium. However, it would be interesting to determine whether other potent RNase inhibitors such as arabinonucleotide ara-Ura-3'P can successfully inhibit the extracellular degradation of RNA without affecting uptake (Pollard and Nagyvary, 1973).

There are conflicting reports on the fate of RNA ingested by

mammalian cells. The ingested RNA may remain stable (Galand et al 1966; Niu et al, 1972; and Holoubek et al, 1966) or be degraded (Shanmugan and Bhargava, 1966; Bausek and Merigan, 1969). The results obtained from different experiments carried out in this project suggest that bacterial rRNA is rapidly degraded both by Ehrlich ascites cells with normal and with high RNase activities to about the same extent. The first experiment compared the amount of radioactive RNA taken up and the radioactivity present as acid-precipitated material in the absence and presence of actinomycin D. Shortly after the incubation of cells with RNA, a large portion of the ingested RNA was degraded to acid-soluble products and the pool of acid-soluble products seems to be utilized for mammalian RNA synthesis. Preliminary results indicated that homologous RNA taken up by Ehrlich ascites cells with normal and high RNase activities is also rapidly degraded and the degradation products are also utilized for RNA synthesis. Since only the difference between the total RNA uptake and the acid precipitated RNA in the presence of actinomycin D was used to estimate the acid-soluble degradation products in the cells, the amount of RNA absorbed on the cell membrane does not interfere with the estimation of RNA degradation by this method.

In order to characterize the labelled RNA which had been ingested by the cells, the RNA was extracted and analysed by polyacrylamide gel electrophoresis. The RNA profiles obtained from the cells incubated continuously with RNA also provide evidence that the ingested RNA is degraded because the increase of RNA in the profiles with time is moderate compared to the total RNA uptake by the cells. This suggests

small to be extracted in the phenol extraction procedure. However, the main obstacle in following the characteristics of ingested RNA was the finding that RNA is degraded extracellularly and the degraded RNA is probably taken up together with the undegraded RNA. The appearance of this partially degraded RNA in the profiles of extracted RNA can be easily mistaken for the result of degradation of ingested RNA.

In order to minimize the complication which might arise from the ingestion of partially degraded RNA, the RNA in the medium was removed after a brief exposure of cells to RNA. The fate of this small amount of relatively intact RNA which was incorporated was then traced by polyacrylamide gel electrophoresis. In addition, the RNA in the medium after the brief incubation period was compared with the RNA extracted from the cells. Therefore, even though a small amount of extracellular degradation occurs during incubation, a comparison of the profiles of intracellular and extracellular RNA should yield information on the fate of the ingested RNA.

From the results obtained in the three different experiments on the fate of the ingested RNA it is clear that the RNA taken up is degraded rapidly to acid-soluble components. The results also show that in the absence of actinomycin D, the heterogeneous nuclear RNA, ribosomal RNA precursors and then the ribosomal RNA are labelled both in cells with normal and high RNase activities. It seems, therefore, that the acid-soluble material is used for RNA synthesis in Ehrlich ascites cells. It is not clear from the results, however, whether intact or degraded RNA is incorporated directly into the mammalian RNA or whether degraded RNA enters the nucleotide pool first before being incorporated.

Various biological effects have been attributed to intact RNA taken up by mammalian cells (Duke et al,1972; Niu et al, 1972; Robertson and Mathew, 1973). Results obtained in this project show that the ingested RNA is rapidly degraded and, therefore, it is not likely that intact RNA is the cause of biological changes unless only a small amount of intact RNA is required to initiate the various reactions. One report suggested that, although degradation of ingested RNA does take place, only the intact or undegraded tRNA is functional in the acylation process (Herrera et al, 1966). On the basis of results obtained in this project, it seems more likely that the biological changes are caused by adsorption of RNA on the cell membrane, especially when high concentrations of RNA are added to the cells (Bausek and Merigan, 1969).

The fate of the ingested RNA seemed to be similar in Ehrlich ascites cells with normal and with high RNase activities. This result was unexpected because the cells with high RNase activities have elevated acid and alkaline RNase II activities which are expressed outside the cells by a rapid degradation of RNA in the medium. It is possible that the elevated RNase activities caused by the actinomycin D treatment can be detected in vitro, but they may not be expressed in vivo. The actinomycin D treatment may change the localization of the enzymes or the enzymes responsible for the intracellular degradation of foreign macromolecules may be similarly located and unaltered in both types of cells. Further experiments should therefore be carried out to compare the localization of the RNases in both types of cells. Another possibility is that the amount of RNA taken up is not great enough to illustrate a difference in the rate of degradation in cells with normal and high RNase activities. The amount of RNA taken up

by these cells is small and sufficient RNase may be present in both types of cells to degrade the ingested RNA rapidly.

# BIBLIOGRAPHY


Amos, H. and K. G. Kearns. 1963. Influence of Bacterial Ribonucleic Acid on Animal Cells in Culture. Exptl. Cell Res. 32: 14.

Bases, R. E. and J. Huppert. 1966. Impairment of EMC Viral Infectivity by Cell-Associated Ribonuclease. Virology 29: 596.

Bausek, G. H. and T. C. Merigan. 1969. Cell Interaction with a Synthetic Polynucleotide and Interferon Production in vitro. Virology 39: 491.

Bhargava, P. M. and G. Shanmugam. 1971. Uptake of Nonviral Nucleic Acid by Mammalian Cells. Progress in Nucleic Acid Res. and Mol. Biol. 11: 103.

Bishop, D.C. and P. Abramhoff. 1965. In vitro Uptake of Isotopically Labelled RNA by Mammalian Spleen Cells. Proc. Soc. Exptl. Biol. Med. 120: 378.

Bray, G. A. 1960. A Simple Efficient Liquid Scintillator for Counting Aqueous Solutions in a Liquid Scintillation Counter. Analytical Biochemistry 1: 279.

Busch, H., T, C, Choi, S. T. Crooke and S. Okada. 1972. Genetic Engineering and Cancer Chemotherapy. Oncology 26: 152.

Caldwell, I. C. and M. F. Chan. 1970. Tumour Cell Metabolism in vitro: A Study of Incubation Media. Can. J. Biochem. 48: 517.

Chakravorty, A. K. and H. Busch. 1967. Alkaline Ribonuclease and Ribonuclease Inhibitor in Nuclear and Nucleolar Preparations from Normal and Neoplastic Tissues. Can Res. 27: 789.

Chin, P. H. and M. S. Silverman. 1967. Studies on the Transfer of Myeloma Protein Synthesis with RNA Isolated from the $C_3H$ Plasma Cell Tumour. I. Uptake of Isotopically Labelled Tumour RNA by Lymphoid Cells. J. of Immunology 99: 476.

Cohn, Z. A. and E. Parks. 1966. The Regulation of Pinocytosis in Mouse Macrophages. J. Exptl. Med. 125: 213.

Colby, C. and M. J. Chamberlin. 1969. The Specificity of Interferon Induction in Chick Embryo Cells by Helical RNA. Proc. Nat. Acad. Sci. U.S.A. 63: 160.

Colter, J. S. 1962. The Uptake of High Molecular Weight Ribonucleic Acid by Cultured Mammalian Cells. Proc. 5th Can. Cancer Res. Conference 5: 65.

Colter, J. S., J. Kuhn and K.A.O. Ellem. 1961. The Ribonucleases of Mouse Ascites Tumours. Cancer Res. 21: 48.

Crestfield, A. M., K. C. Smith and F. W. Allen. 1955. The Properties and Characterization of Ribonucleic Acids from Yeast. J. Biol. Chem. 216: 185.

Crooke, S. T., O Okada and H. Busch. 1972. A Surface 3'-Exonuclease of Novikoff Hepatoma Ascites Cells. Cancer Res. 32: 1745.

Culp, L. A. and G. M. Brown. 1968. Transfer Ribonucleic Acid Methylases of HeLa Cells. Arch. Biochem. Biophys. 124: 483.

Darnell, J. E. Jr. 1968. Ribonucleic Acids from Animal Cells. Bacteriological Reviews 32: 262.

de Duve, C. 1963. The Lysosomes. Ciba Foundation Symposium. Edited by A.V.S.de Reuch and M.P.Cameron, page 1.

de Lamirande, G. and C. Allard. 1959. Studies on the Distribution of Intracellular Ribonucleases. Annals New York Academy of Sciences 81: 570.

Dickman, S. R. and G. A. Morrill. 1959. Intracellular Localization and Chromatography of Mouse Pancreas Ribonucleases. Annals New York Academy of Sciences 81: 585.

Duke, L. J., C. Miller and S. Harshman. 1972. Induction of Antibody Synthesis by Purified Immunogenic RNA in Vitro. Nature New Biology 235: 180.

Easty, D. M., L. Ledoux and E. J. Ambrose. 1956. The Action of Ribonuclease on Neoplastic Growth 3: Studies by Interference Microscopy. Biochim. Biophys. Acta 20: 528.

Erbe, E., J. Preiss, R. Seifert and H. Hilz. 1966. Increase in RNase and DPNase Activities in Ascites Tumour Cells Induced by Various Cytostatic Agents. Biochem. Biophys. Res. Commun. 23: 392.

Galand, P. and L. Ledoux. 1966. Uptake of Exogenous Ribonucleic Acids by Ascites Tumour Cells. Exptl. Cell Res. 43: 391.

Galand, P., J. Remy and L. Ledoux. 1966. Uptake of Exogenous Ribonucleic Acid by Ascites Cells. Exptl. Cell Res. 43: 381.

Girija, N. S. and A Sreenivisan. 1966. Characterization of Ribonucleases and Ribonuclease Inhibitor in Subcellular Fractions from Rat Adrenals. Biochemical J. 98: 562.

Giudice, G. A. M. Pirrone, M. Roccheri and M. Trapani. 1973. Maturation Cleavage of Nucleolar Ribosomal RNA Precursor can be Calalyzed by Non-specific Endonuclease. Biochim. Biophys. Acta 319: 72.

Heppel, L. A. 1966. Pig Liver Nuclei Ribonuclease 1. Procedures in Nucleic Acid Research. Edited by G.L.Cantoni and D.R.Davies. Harper and Row Publishers. New York and London, page 31.

Herrera, F., R. H. Adamson and R. C. Gallo. 1970. Uptake of Transfer Ribonucleic Acid by Normal and Leukemic Cells. Proc. Nat. Acad.Sci. U.S.A. 67: 1943.

Hirsch, C. A. and H. H. Hiatt. 1966. Turnover of Liver Ribosomes in Fed and in Fasted Rats. J. Biol. Chem. 241: 5936.

Holoubek, V., L. Fanshier and T. T. Crocker. 1966. The Inhibition on Nuclear RNA Synthesis by Added RNA. Exptl. Cell Res.44:362.

Houssais, J. F. and G. Attardi. 1966. High Molecular Weight Non-ribosomal Type Nuclear RNA and Cytoplasmic Messenger RNA in HeLa Cells. Proc. Nat. Acad. Sci. U.S.A. 56: 616.

Imsande, J. and B. Ephrussi. 1964. Reevaluation of Assays Used to Show RNA Induction of Glucose-6-Phosphatase in Ascites Cells. Science 144: 854.

Juliano, J. and E. Mayhew. 1972. Interaction of Polynucleotides with Cultured Mammalian Cells. 1. Uptake of RNA by Ehrlich Ascites Carcinoma Cells. Exptl. Cell Res. 73: 3.

Kay, J. E. and H. L. Cooper. 1969. Rapid Labelled Cytoplasmic RNA in Normal and Phytohaemagglutinin-Stimulated Hyman Lymphocytes. Biochim. Biophys. Acta 186: 62.

Kelly, D. E. and R. P. Perry. 1971. The Production of Ribosomal RNA from High Molecular Weight Precursors. 11. Demonstration of an Exonuclease in Isolated Nucleoli. Biochim. Biophys. Acta 238: 357.

Koenig, H. 1964. Studies of Brain Lysosomes. 1. Subcellular Distribution of Five Hydrolases, Succinate Dehydrogenase and Gangliosides in Rat Brain. J. Neurochem. 11: 729.

Kraft, N. and Shortman, K. 1970. A Suggested Control Function for the Animal Tissue Ribonuclease - Ribonuclease Inhibitor System Based on the Studies of Isolated Cells and Phytohaemagglutinin-Transformed Lymphocytes. Biochim. Biophys. Acta 217: 164.

Kubinski, H., Gibbs, P. and C. B. Kasper. 1972. Interaction of Nucleic Acids and Cellular Membranes and Behaviour of Membranes in CsCl Density Gradients. Biochim. Biophys. Acta 281: 244.

Lazarus, H. M. and M. B. Sporn. 1967. Purification and Properties of a Nuclear Exoribonuclease from Ehrlich ascites Tumour Cells. Proc. Nat. Acad. Sci. U.S.A. 57: 1386.

Ledoux, L. 1956. Action of Ribonuclease on Neoplastic Growth. 11. Action on Lanschutz Ascites Cells in vitro. Biochim. Biophys. Acta 20: 369.

Mayhew, E. and R. Juliano. 1973. Interaction of Polynucleotides with Cultured Mammalian Cells.11. Cell Surface Charge Density and RNA Uptake. Exptl. Cell Res. 77: 409.

Morais, R. and G. de Lamirande. 1965. Formation of Oligonucleotides with 5'-Phosphomonoester End Group by Rat-Liver Microsomal Fraction. Biochim. Biophys. Acta 103: 506.

Mirault, M. E. and K. Scherrer. 1972. In vitro processing of HeLa Cell Pre-ribosomes by a Nucleolar Endoribonuclease. Fed. Eur. Biochem. Soc. Letters 20: 233.

Niu, M. C. 1963. The Mode of Action of Ribonucleic Acid. Develop. Biology 7: 379.

Niu, M. C., C. C. Cordova, L. C. Niu. 1961. Ribonucleic Acid-Induced Changes in Mammalian Cells. Proc. Nat. Acad. Sci. U.S.A. 48: 1964.

Niu, M. C., L. C. Niu and A. Guha. 1968. The Entrance of Exogenous RNA into Mouse Ascites Cells. Proc. Soc. Exptl. Biol. and Med. 128: 550.

Niu, M. C., T. Lin and L. C. Niu. 1972. Antagonistic Action of Exogenous Histone and RNA in Mouse Ascites Cells. Proc. Soc. Exptl. Biol. and Med. 140: 256.

Peacock, A. C. and C. W. Dingman. 1968. Molecular Weight Estimation and Separation of RNA by Electrophoresis in Agarose-Acrylamide Composite Cells. Biochemistry 7: 668.

Pene, J. J., E. Knight, Jr. and J. E. Darnell, Jr. 1968. Characterization of a New Low Molecular Weight RNA in HeLa Cell Ribosomes. J. Mol. Biol. 33: 609.

Pollard, D. R. and J. Nagyvary. 1973. Inhibition of Pancreatic Ribonuclease A by Arabinonucleotides. Biochemistry 12: 1063.

Prestayko, A. W., B. C. Lewis and H. Busch. 1972. Endoribonuclease Activity Associated with Nucleolar Ribonucleoprotein Particles from Novikoff Hepatoma. Biochim. Biophys. Acta 269: 90.

Prestayko, A. W., B. C. Lewis and H. Busch. 1973. Purification and Properties of a Nucleolar Endoribonuclease from Novikoff Hepatoma. Biochim. Biophys. Acta 319: 323.

Razzell, W. E. 1961. Partial Purification and Properties of Phosphodiesterase I from Hog Kidney. J. Biol. Chem. 236: 3031.

Razzell, W. E. 1963. Phosphodiesterases. Methods in Enzymology 6, ed. by S.P.Colowick and N.O.Kaplan. Academic Press Inc., New York, page 236.

Razzell, W. E. 1967. Polynucleotidases from Animal Tissues. Experientia 23: 321.

Razzell, W. E. and H. G. Khorana. 1961. Studies on Polynucleotides. 10. Enzymic Degradation. Some Properties and Mode of Action of Spleen Phosphodiesterase. J. Biol. Chem. 236: 1144.

Robertson, H. D. and M. B. Mathews. 1973. Double-Stranded RNA as an Inhibitor of Protein Synthesis and as a Substrate for a Nuclease in Extracts of Krebs II Ascites Cells. Proc. Nat. Acad. Sci. U.S.A. 70: 225.

Roth, J. S. 1956. Studies on the Properties and Distribution of Ribonuclease Inhibitor in the Rat. Exptl. Cell Res. 21: 34.

Roth, J. S. 1957. Ribonuclease. 6. Partial Purification and Characterization of the Ribonucleases of Rat Liver Mitochondria. J. Biol. Chem. 227: 591.

Roth, J. S. 1959. Comparative Studies on Tissue Ribonucleases. Annals New York Academy of Sciences 81: 611.

Roth, J. S. 1962. Ribonuclease. 9. Further Studies on Ribonuclease Inhibitor. Biochim. Biophys. Acta 61: 903.

Roth, J. S. 1963. Ribonuclease Activity and Cancer. Cancer Res. 23: 657.

Schell, P. L. 1971. Uptake of Polynucleotides by Intact Mammalian Cells. 8. Synthetic Homoribopolynucleotides. Biochim. Biophys. Acta 240: 472.

Schwarz, M. R. and W. O. Reike. 1962. Appearance of Radioactivity in Mouse Cells after Adminstration of Labelled Macromolecular RNA. Science 136: 152.

Shanmugam, G. and P. M. Bhargava. 1966. The Uptake of Homologous Ribonucleic Acid by Rat Liver Parenchymal Cells in Suspension. Biochemical J. 99: 297.

Sheppard, E. P., K. Hogan and K. B. Roberts. 1970. Increase in Acid Stable Ribonuclease Activity in Fasted Rat Liver. Biochim. Biophys. Acta 217: 159.

Scherrer, K., H. Latham and J. E. Darnell. 1963. Demonstration of an Unstable Ribonucleic Acid and of a Precursor to Ribosomal Ribonucleic Acid in HeLa Cells. Proc. Nat. Acad. Sci.U.S.A.49: 240.

Shortman, K. 1961. Studies on Cellular Inhibitors of Ribonuclease. 1. The Assay of the Ribonuclease-Inhibitor System, and the Purification of the Inhibitor from Rat Liver. Biochim. Biophys. Acta 51: 37.

Sirakov, L. M. and C. D. Kochakian. 1969. The Stability of Ribonuclease Inhibitor from Guinea-Pig Liver. Biochim. Biophys. Acta 195: 572.

Sporn, M. B., H. M. Lazarus, J.M. Smith and W. R. Henderson. 1969. Studies on Nuclear Exoribonucleases. 3. Isolation and Properties of the Enzyme from Normal and Malignant Tissues of the Mouse. Biochemistry 8: 1698.

Utsunomiya, T. and J. S. Roth. 1966. Studies on the Function of Intracellular Ribonucleases. 5. Ribonuclease Activity in Ribosomes and Polysomes Prepared from Rat Liver and Hepatomas. J. Cell Biol. 29: 395.

von Tigerstrom, R. G. 1972. Changes in Ribonucleic Acid Degrading Enzymes in Ehrlich Ascites Cells During and After Actinomycin D Treatment. Can. J. Biochem.50: 244.

von Tigerstrom, R. G. 1973. The Degradation of Newly Synthesized RNA in Ehrlich Ascites Cells. Can. J. Biochem.51: 495.

Weinberg, R. A. 1973. Nuclear RNA Metabolism. Ann. Rev. Biochem. 42: 329.

Weinberg, R. A. and S. Penman. 1970. Processing of 45S Nucleolar RNA. J. Mol. Biol. 47: 169.

Yoon, C. H. 1965. Incorporation of Exogenous RNA into Nelson's Ascites Cells *in vitro*. Exptl. Cell Res. 38: 386.